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INTRACEREBROVENTRICULAR PROPIONIC ACID INCREASES LOCOMOTOR ACTIVITY AND NOSE POKES IN RATS TESTED IN AN AUTOMATED HOLE-BOARD APPARATUS: DOSE RESPONSE EFFECTS IN AN ANIMAL MODEL OF AUTISM SPECTRUM DISORDERS

Melissa M. Meeking

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ABSTRACT AND KEYWORDS

Autism spectrum disorders (ASD) are a cluster of neurodevelopmental disorders characterized by abnormal social interactions, communication deficits, and repetitive and restricted behaviours. Propionic acid (PPA), a component of fatty acid metabolism, is endogenous to the human body, produced by enteric gut bacteria, and crosses both the gut-blood and the blood-brain barrier. Previous research has demonstrated that repeated intracerebroventricular (ICV) infusions of PPA in adult rats produce behavioural and neuropathological changes similar to those seen in ASD patients. The current studies further characterized this animal model, focusing on repetitive and perseverative behaviours, through the use of the hole-board apparatus. Adult male Long-Evans rats received ICV infusions twice a day, 4 hours apart, of high-dose PPA (0.26 M), low-dose PPA (0.052 M), or phosphate buffered saline (PBS) for 7 consecutive days. Locomotor activity and nose poke behaviour were recorded daily in an automated open field apparatus (Versamax) equipped with a 16 well hole-board for 30 minutes immediately after the second infusion. In the first study, all wells remained empty. PPA treatment increased locomotor and nose poking behaviour in a dose-dependent manner. In the second study, relevant olfactory stimuli (i.e., clean and soiled bedding) was placed within the wells as a means of measuring perseverative behaviour. Both high- and low-dose PPA animals failed to modify their nose poking patterns, suggestive of perseverative behaviour. This work further supports the face validity of the PPA rodent model of ASD by demonstrating that repetitive and perseverative behaviours, core symptoms of autism, occur within PPA infused rats.

Keywords: autism spectrum disorder, hole-board apparatus, locomotor activity, perseveration, propionic acid, repetitive behaviour

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I did it.

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LIST OF ABBREVIATIONS

ADHD:	Attention deficit hyperactivity disorder
ANOVA:	Analysis of variance
ASD:	Autism spectrum disorders
BBB:	Blood brain barrier
BDNF:	Brain-derived neurotrophic factor
CNS:	Central nervous system
CREB:	Cyclic AMP response-element-binding
GI:	Gastrointestinal
ICV:	Intracerebroventricular
IL:	Interleukin
MMR:	Mumps-measles-rubella
OCD:	Obsessive compulsive disorder
PBS:	Phosphate buffered saline
PPA:	Propionic acid
SEM:	Standard error of the mean
SCFA:	Short-chain fatty acids
TNF:	Tumour necrosis factor

Chapter 1

General Introduction

1.1 Autism Spectrum Disorders

Autism spectrum disorders (ASD) are a heterogeneous family of neurodevelopmental disorders characterized by three core symptom domains: deficits in communication, abnormal social interaction, and restricted and/or repetitive interests and behaviours (American Psychiatry Association, 1994). Other symptoms include hyperactivity, sensitivity to sensory stimuli, resistance to change, and cognitive deficits in attention or memory (Kootz, Marinelli, & Cohen, 1982; Markram, Rinaldi, & Markram, 2007; Murray, 2010; Sasson, Turner-Brown, Holtzclaw, Lam, & Bodfish, 2008). Furthermore, autism often co-exists with a variety of other conditions, including fragile X syndrome, tuberous sclerosis, mental retardation, and epilepsy (Muhle, Trentacoste, & Rapin, 2004; Tuchman, Moshe, & Rapin, 2009). Typically, autistic symptoms become evident in children before 36 months of age (Rapin, 1997). Most children who develop autism have early onset symptoms within the first year of life characterized by abnormal social development (e.g., deficits in social smiling and eye contact), delay in babbling, poor motor coordination, and repetitive motor actions (Barbaro & Dissanayake, 2009; Landa, 2008). About one-third of cases are considered late onset. Children appear to develop normally until about 2 years of age, at which point social and communication skills regress to that of an autistic phenotype (Ozonoff, Williams, & Landa, 2005)

Over the last decade, there has been nearly a 300% increase in the prevalence of autism, with current estimates of approximately 1 in every 110 children affected with autism, and males are four times more likely to be affected than females (Boyle et al., 2011). Although the significant increase in ASD is at least partly due to better awareness

and broadening diagnostic criteria, it is unlikely that this can solely account for the substantial increased prevalence of autism. In fact, several recent studies have provided support for a true increase in disease frequency over the last several decades, unrelated to changes in diagnostic criteria (Blaxill, 2004; Schechter & Grether, 2008). The rising incidence of autism is a matter of urgent public concern. Unfortunately, the etiology of autism is poorly understood and researchers do not fully understand the contributions of genetics, biology, and the environment to the pathogenesis and on-going symptomatology of ASD. Thus, it is imperative that research examines the mechanisms behind autism in order to better understand this complex disease. An effective way to accomplish this is to develop an animal model. Animal models allow researchers to examine the underlying mechanism/cause of a disease in a controlled manner that is ethically impossible to do in humans. The major purpose of the current studies is to provide further support for a rodent model of autism developed in our laboratory as a means of furthering our understanding of the underlying pathophysiology of ASD.

1.1.1 Genetic and Environmental Aspects of ASD

Evidence from family and twin studies suggests a moderate to strong genetic basis for ASD susceptibility. The reported heritability estimate for autism is as high as 90%, with a significantly higher concordance rate in monozygotic twins than dizygotic twins (Bailey et al., 1995; Hallmayer et al., 2011). Moreover, there is a high recurrence rate of autism such that in families with at least one case of ASD, the prevalence of autism within the family is 100 times higher than the general population (Rutter, Silberg, O'Connor, & Simonoff, 1999). Autism is considered biologically and phenotypically heterogeneous, and this variability is also apparent in genetic linkage

studies which show numerous candidate genes in ASD populations (Geschwind, 2011). Unfortunately, there is no single mutation attributed to causing ASD and no genes that can serve as definitive biomarkers for the disorder (Scherer & Dawson, 2011). Thus, there is a general agreement in the literature that ASD has a large genetic component, but further research is required to translate genomic linkage discoveries into diagnostics relevant for prevention and treatment.

Because genetics cannot fully account for the manifestation of autism, researchers have become increasingly interested in environmental agents that may act during critical developmental periods. Chemical toxins and infectious agents present in the environment during prenatal and early postnatal periods have been implicated in the pathogenesis of ASD. For instance, children exposed to thalidomide, valproic acid, and ethanol in utero have an increased risk of ASD (Ratajczak, 2011). Maternal infections have also been identified as a risk factor for autism, with approximately 43% of mothers with an autistic child experiencing a respiratory, urinary, or other infection during pregnancy as compared to 26% of control mothers (Comi, Zimmerman, Frye, Law, & Peeden, 1999). Fever, a common physiological response to infection, may be treated with anti-pyretics, such as acetaminophen, during pregnancy. Anti-pyretics interfere with normal immunological development in the brain, and may partially explain why maternal infection during pregnancy leads to increased susceptibility to ASD in offspring (Currenti, 2010). Maternal stress, maternal anti-bodies, heavy metals, phthalates, organophosphate pesticides, and many other environmental factors are also believed to play a role in the etiology of ASD (Ratajczak, 2011; Ronald, Pennell, & Whitehouse, 2010). Although previously thought to be a causal environmental factor in

ASD, numerous studies have failed to support a relationship between the mumps-measles-rubella (MMR) vaccine and autism (Honda, Shimizu, & Rutter, 2005; Hornig et al., 2008; Schultz et al., 2008). It is likely that an interaction between genetic predisposition and environmental factors results in the manifestation and severity of ASD. Indeed, a growing number of environmentally responsive genes and pathways are being identified in some autistic populations (Fatemi, 2008; Herbert, 2010)

1.1.2 Central Nervous System and Systemic Abnormalities in ASD

Although autism has been traditionally considered a strongly brain-based disorder, emerging findings support the idea of ASD as a systemic disorder involving immune and metabolic dysfunction that also affects the central nervous system (CNS) (Chauhan & Chauhan, 2006; Herbert, 2005a). In particular, there is increasing evidence for the role of oxidative stress, antioxidant impairment, and chronic inflammatory processes in ASD from childhood through adulthood (Anderson, Hooker, & Herbert, 2008). Recent studies of post-mortem brain tissue in patients with ASD revealed innate neuroinflammatory changes characterized by reactive astrogliosis and activated microglia in the cerebral cortex, white matter, and cerebellum (Morgan et al., 2010; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). Cytokine protein arrays also demonstrated elevations in proinflammatory cytokines , such as interleukin-6 (IL-6), in both the brain tissue and cerebrospinal fluid of autistics. Interestingly, these neuroinflammatory changes were observed in both adults and children with the disorder, suggesting that neuroinflammation may be a permanent state of pathology in ASD that starts early. This immune dysfunction may be a potential mechanism for the pathogenesis of autism, given that glial cells are important in developing and mature

nervous systems via their role in immune function, support of neurons, the integrity of the blood-brain barrier (BBB), and myelination of neurons (Barron, 1995).

Besides an innate neuroinflammatory response and increased cytokines, other CNS abnormalities have also been found in persons with ASD. Imaging and post-mortem studies have demonstrated that the brain of autistics is grossly normal, but other subtle anatomic aberrations do exist (Bauman & Kemper, 2005). Young children with autism (but not older) have unusually large brain volumes due to an overgrowth shortly after birth followed by slowed growth a few years later (Herbert, 2005b). Compared to controls, autistic brains show smaller neuronal cell size and increased cell packing density in the limbic system (e.g. hippocampus and amygdala), a pattern consistent with truncated development (Palmen, van Engeland, Hof, & Schmitz, 2004). Purkinje cell size in the cerebellum has been shown to be consistently reduced in brain tissue of ASD patients at autopsy (Fatemi et al., 2002). Additionally, elevated 3-nitrotyrosine levels (a specific marker of oxidative damage to proteins), has been documented in cerebellar tissue in autism (Sajdel-Sulkowska, Lipinski, Windom, Audhya, & McGinnis, 2008). Imaging studies of autistics have also shown an overall increase in white matter thickness, suggestive of an underlying glial dysfunction, as well as a widespread reduction in the functional connectivity among brain regions (Hughes, 2007; Sokol & Edwards-Brown, 2004; Stigler, McDonald, Anand, Saykin, & McDougle, 2011).

Various systemic abnormalities indicative of altered immune regulation and oxidative stress have also been observed in autism. An association between ASD and immune dysfunction has been supported by reports of increased incidence of autoimmune disease in families with an autistic child and altered metabolism in immune

cells in children diagnosed with ASD (Atladottir et al., 2009; Suh, Walsh, McGinnis, Lewis, & Ames, 2008). Furthermore, analysis of plasma samples from autistic children, as compared to controls, have shown elevated production of brain-derived neurotrophic factor (BDNF) and tumour necrosis factor alpha (TNF-α) by peripheral immune cells under mitogenic stimulation, suggestive of an excessive innate immune response (Enstrom et al., 2008; Jyonouchi, Sun, & Le, 2001). Oxidative stress has also been implicated in ASD pathogenesis. At a cellular level, oxidative stress occurs when the levels of deleterious reactive oxygen species exceed the antioxidant and detoxification capabilities of that cell, which can result in mitochondrial dysfunction, abnormal membrane lipids, inflammation, and DNA damage (Pardo & Eberhart, 2007). Indeed, increased oxidative stress has been observed in autism, as demonstrated by increased lipid peroxidation and decreased antioxidant proteins (e.g., glutathione and transferrin) in the plasma of children with autism (Chauhan, Chauhan, Brown, & Cohen, 2004; James et al., 2006). Recent evidence has also suggested a role for metabolic dysfunction in autism involving disordered fatty acid profiles and altered energy dynamics of the mitochondria (Gargus & Imtiaz, 2008; Wiest, German, Harvey, Watkins, & Hertz-Picciotto, 2009).

1.1.3 Gastrointestinal Factors of ASD

Although a gastrointestinal (GI) pathology specific and unique to autism has not been identified, there appears to be a high degree of comorbidity between gastrointestinal disorders and ASD (Buie et al., 2010). A number of GI symptoms and/or disorders have been reported in ASD patients, including diarrhea, abdominal pain, constipation, bloating, gastroesophageal reflux, gastritis, inflammatory bowel disease,

and celiac disease, among others (Bauman, 2010). Dietary factors are also associated with the behavioural symptoms of ASD. Parents frequently report an increase in the severity of autistic symptoms in their child following the consumption of refined wheat and dairy products, and an improvement in symptoms following the implementation of a casein- and gluten-free diet (Jyonouchi, 2009). In support of these anecdotal reports, a recent randomized controlled trial showed improvements in attention and reduced hyperactivity in children with ASD after a gluten- and casein-free dietary intervention (Whiteley et al., 2010). Moreover, some children with ASD have intestinal lesions, increased intestinal permeability, and pro-inflammatory cytokine responses against dietary proteins similar (but not identical) to those observed in patients with known dietary protein intolerance/allergy (de Magistris et al., 2010; Jyonouchi, Sun, & Itokazu, 2002; White, 2003).

The human intestinal tract contains an enormous microbial ecosystem, or gut microbiota, that is partially responsible for maintaining human health via protective, structural, and metabolic roles (Prakash, Rodes, Coussa-Charley, & Tomaro-Duchesneau, 2011). Disruption of the normal gut microbiota has been associated with various disease processes, including autoimmune and metabolic disorders (Mai & Draganov, 2009). Analysis of stool specimens from autistic children with co-morbid GI symptoms has shown evidence of abnormal gut microflora, particularly a greater diversity and number of clostridial species (Finegold et al., 2002; Parracho, Bingham, Gibson, & McCartney, 2005; Song, Liu, & Finegold, 2004). Another enteric organism, *Desulfovibrio*, has also been shown to be more common in regressive cases of autism (Finegold, 2011). Colonization with clostridial or other species may be facilitated by

antibiotic use, which can disrupt the indigenous gut species and promote the overgrowth of antibiotic resistant pathogenic organisms (as is the case with both *Clostridium* and *Desulfovibrio*). Indeed, a retrospective study by Niehus and Lord (2006) found that the early medical history of children with ASD included significantly more antibiotic use than typically developing children. Recent evidence from studies in rodents has shown that gut microbiota can modulate brain development and behaviour (Heijtz et al., 2011; Li, Dowd, Scurlock, Acosta-Martinez, & Lyte, 2009). Abnormal gut microflora may contribute to the pathogenesis of ASD in genetically sensitive subpopulations.

1.2. Propionic Acid

Propionic acid (PPA) is a component of fatty acid metabolism, and like other short-chain fatty acids (SCFA), such as acetic and butyric acid, PPA is a metabolite of microbial fermentation of undigested food in the colon (Al-Lahham, Peppelenbosch, Roelofsen, Vonk, & Venema, 2010). Besides gut bacterial production, which contributes to about 20% of PPA sources in the body, PPA is also produced as a result of amino acid (~50%) and odd-chain fatty acid (~30%) catabolism (Thompson et al., 1990). SCFAs comprise a significant metabolic energy source, and the metabolism of PPA, via a series of steps, results in succinyl-CoA which can be incorporated directly into the Krebs cycle (Al-Lahham et al., 2010). Certain families of enteric bacteria, including propionibacteria and clostridia, are known to produce PPA (Fernandez-Garcia & McGregor, 1994; Finegold, Song, & Liu, 2002). In addition to these endogenous sources, PPA is present in some food products that use bacterial fermentation in the manufacturing process (e.g. dairy products, such as cheese and yogurt) and is commonly used as a food preservative due to its anti-fungal properties (Brock & Buckel, 2004).

However, it is important to note that these food sources contribute to only a small proportion of total PPA in the human circulation due to the large quantities produced by bacterial fermentation in the gut (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987).

PPA has direct physiological effects on the GI tract, including altering gut motility, increasing the contraction of colonic smooth muscle, dilating colonic arteries, and activating mast cells (Karaki et al., 2006; Mitsui, Ono, Karaki, & Kuwahara, 2005). As a weak organic acid, PPA can readily cross both the gut-blood and blood-brain barriers passively or actively via monocarboxylate transporters where it can cross cell membranes and accumulate within cells (Bergersen, Rafiki, & Ottersen, 2002). PPA is capable of inducing widespread effects in the CNS, and can inhibit Na^+/K^+ ATPase, increase NMDA receptor sensitivity, alter mitochondrial and fatty acid metabolism, and affect immune activation and gene expression (Brass & Beyerinck, 1988; de Mattos-Dutra et al., 2000; Parab, Nankova, & La Gamma, 2007; Wajner, Latini, Wyse, & Dutra-Filho, 2004; Wyse et al., 1998). Furthermore, PPA can concentrate within cells, resulting in intracellular acidification, which can alter neurotransmitter release, inhibit gap junctions, and promote intracellular calcium release, all of which can potentially affect neuronal communication and behaviour (Remblier, Pontcharraud, Tallineau, Piriou, & Huguet, 1999; Rorig, Klausa, & Sutor, 1996). Given the diverse physiological effects on the GI tract and CNS, PPA may be a putative link between the dietary, GI, and behavioural symptoms of ASD in genetically susceptible subpopulations.

Several findings suggest that PPA may be associated with the etiology and pathogenesis of ASD. Propionic acidemia is a genetically acquired metabolic disorder

which results in an accumulation of PPA caused by a deficiency of the mitochondrial enzyme propionyl-coenzyme A carboxylase (Hofherr et al., 2009). Symptoms of propionic acidemia include vomiting, loss of appetite, and motor, social, and language delay, all of which are reminiscent of autistic symptoms (Kaya et al., 2008). In addition, exposure to valproic acid may lead to increased levels of SCFAs such as PPA, and valproic acid exposure early in development increases the likelihood of ASD (Ornoy, 2009). As previously mentioned, some children with autism, particularly those with GI symptoms and behavioural regression, have increased levels of enteric *Clostridium* and *Desulfovibrio*, both of which are known to increase levels of PPA and other SCFAs (Finegold, 2011). Additionally, serum studies from autistic patients have shown metabolic dysfunction consistent with PPA's physiological effects, such as impairments of glutathione and carnitine metabolism (Filipek, Juranek, Nguyen, Cummings, & Gargus, 2004; James et al., 2006).

The properties of PPA led our laboratory to propose that alteration of PPA levels or metabolism may be related to ASD, and therefore we recently investigated the effects of exposure to PPA in rodents as a potential novel animal model of ASD. Repeated, central infusions of PPA in adult rats produced behavioural, biochemical, electrophysiological, and neuropathological effects similar to those seen in human cases of autism (MacFabe et al., 2007, 2008). We found that intracerebroventricular (ICV) infusions of PPA in adult rats induced repetitive behaviours, kindled seizures, hyperactivity, turning behaviour, and retropulsion, all of which bear resemblance to symptoms seen in ASD (MacFabe et al., 2007, 2008). Immunohistochemical analyses of brain tissue in PPA-infused rodents revealed an innate neuroinflammatory response

characterized by reactive astrogliosis and activated microglia, as well as increased oxidative stress and decreased glutathione, in the absence of any apoptotic cell loss (MacFabe et al., 2007, 2008), which is consistent with autopsy studies of human autistic patients (Bauman & Kemper, 2005; Vargas et al., 2005). Furthermore, analysis of brain lipid composition in PPA-infused rodents reveals altered phospholipid and acylcarnitine profiles similar to the abnormal lipid profiles seen in erythrocytes and plasma of autistic patients (Bell et al., 2004; Thomas et al., 2010)

Another set of studies found that central administration of PPA resulted in social, cognitive, and sensorimotor impairments (Shultz et al., 2008, 2009), lending further credence to the face validity of the PPA rodent model. Given the developmental and systemic nature of ASD, more recent work in our laboratory using the rodent model has evaluated both central and systemic effects of PPA in adolescent and neonatal rats. Thus far, our results have been consistent with symptoms observed in autistic patients, such as object fixation in adolescent rats reminiscent of the restricted interests in ASD (for review see Foley, Tichenoff, Ossenkopp, & MacFabe, 2010; MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011; Shams, Kavaliers, Foley, Ossenkopp, & MacFabe, 2009). Finally, deuterium tracing of PPA in rat brain has allowed our laboratory to confirm PPA associated oxidative stress and edema (Nie et al., 2011). Taken altogether, these findings suggest that alterations in PPA metabolism may play a significant role in the pathogenesis of ASD, and supports the use of PPA in rodents as an effective model of ASD.

1.3 Present Studies

One of the core symptom domains of ASD is the presence of stereotyped or repetitive/restrictive behaviours (American Psychiatry Association, 1994). These behaviours typically occur with high frequency and persist across the lifespan, at higher rates and intensities than any other developmental disability (Esbensen, Seltzer, Lam, & Bodfish, 2009; Matson, Dempsey, & Fodstad, 2009). Repetitive behaviour in ASD may include motor stereotypies (e.g., hand flapping or body rocking), verbal rituals (e.g., repetitive questioning), and sometimes self-injurious behaviours (McDougle, Kresch, & Posey, 2000; Muthugovindan & Singer, 2009). Restricted interests and resistance to change (or perseverative behaviours) also characterize autism and may manifest as inflexible adherence to routines or rituals, a limited number of interests and activities, a behavioural need to preserve "sameness," or preoccupation with part of an object or objects (Kootz, Marinelli, & Cohen, 1982; Matson, Dempsey, & Fodstad, 2009).

Repetitive behaviours are apparent in a wide range of psychiatric disorders, including obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD) Tourette's syndrome, and schizophrenia (Ridley, 1994), as well as in children suffering from sensory impairments or severe intellectual disability (Barry, Baird, Lascelles, Bunton, & Hedderly, 2011). Some of these disorders, such as OCD and ADHD, are often co-morbid with autism (Leyfer et al., 2006). Furthermore, autism spectrum traits have been found in children and adolescents diagnosed with OCD (Ivarsson & Melin, 2008), suggesting that perhaps these disorders have common etiologies, underlying biological mechanisms, or similarly affected brain regions.

Although several studies have shown that dysfunction in the basal ganglia, frontal cortex, and frontostriatal circuits are involved in the manifestation of repetitive and perseverative behaviours seen in many psychiatric disorders, it is important to point out that the repetitive and "sameness" behaviours seen in ASD are characteristically different from those seen in OCD (Ridley, 1994; McDougle et al., 2000). Compared to adults with OCD, autistic patients are more likely to engage in repetitive ordering, hoarding, telling or asking, tapping, and self-damaging behaviours and are less likely to engage in cleaning, checking, and counting behaviours that are common in patients with OCD (McDougle et al., 1995; Zandt, Prior, & Kyrios, 2009). Additionally, adults with autism had significantly more repetitive behaviours than repetitive thoughts, and no autistic patient had repetitive thoughts alone. Few autistic patients made an active effort to suppress or resist their repetitive behaviours. In contrast, nearly all patients with OCD had both repetitive thoughts and behaviours, only a very small percentage (~ 0.5%) had only repetitive behaviours, and 95% of OCD patients regarded the obsessions and compulsions as intrusive and actively attempted to suppress the thoughts and resist the behaviours.

The purpose of the current studies is to further explore the core symptom domain of repetitive and perseverative behaviours in ASD in the context of an animal model. Animal models provide an opportunity to advance our understanding of the biological mechanisms underlying autistic disorders (van der Staay, 2006). In order to extend the validity of the PPA rodent model of ASD proposed by our laboratory, it is essential to test the model using behavioural assays relevant to the symptoms of autism (Crawley, 2007; MacFabe et al., 2007). One such behavioural task that is useful for assessing

repetitive behaviour in rodents is the hole-board apparatus (File & Wardill, 1975; Makanjuola, Hill, Maben, Dow, & Ashcroft, 1977; Moy et al., 2008). The hole-board apparatus is an elevated platform with 16 equally spaced holes (with wells underneath) that can be fitted into an open field chamber, allowing for measurement of both locomotor activity and nose pokes (the measure specific to the hole-board). Although not currently in widespread use as a behavioural test, research has shown that analysis of nose pokes within the hole-board task can provide a valid and reliable measure of exploratory and repetitive behaviours (File & Wardill, 1975a, 1975b). Furthermore, Moy and colleagues (2008) demonstrated that using the hole-board task may be a useful method of assessing restricted and perseverative behaviours in rodents, particularly when relevant olfactory stimuli are placed in the hole-board.

The aims of the current studies were to:

I. Assess repetitive and perseverative behaviour following central PPA treatment using the hole-board task

II. Investigate the dose-response effects of PPA with a low-dose of PPA (0.052 M) and a high-dose of PPA (0.26 M)

Both objectives were devised to further validate the use of PPA as an effective rodent model of ASD. The present studies are the first to investigate the effects of PPA using the hole-board task, and the first to assess dose-related behavioural effects of PPA treatment using a low-dose of PPA not previously used and comparing it to the "high-dose" of PPA used in our previous experiments. Chapter 2 reports the dose-related effects of central administration of PPA on locomotor and nose poking behaviour in adult rats when all wells within the hole-board were empty. It was expected that similar

to previous work, PPA treatment would result in increased locomotor and repetitive activity in a dose-dependent manner. The experiment in Chapter 3 was designed to determine if placement of relevant olfactory stimuli (i.e., soiled and clean cage bedding) within the hole-board would alter the pattern of hole-board exploration and whether PPA-treated animals would adapt their behaviour in response to a change in the hole-board environment. Given that PPA-treated animals have shown a perseverative pattern of responding in the water maze task (Shultz et al., 2009), it was expected that PPA-treated rodents in the hole-board task would fail to adapt their behaviour and that these perseverative effects would be more pronounced in high-dose PPA animals than low-dose PPA animals. Chapter 4 provides a summary of both experiments as well as implications and directions for future research.

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Chapter 2

Intracerebroventricular propionic acid results in a dose-dependent increase in locomotor and nose poking behaviour in rats tested in an automated hole-board apparatus: Support for an animal model of autism spectrum disorders

2.1 Introduction

Autism spectrum disorders (ASD) are a cluster of neurodevelopmental disorders affecting approximately 1 in 110 children, which represents a 300% increase in prevalence over the last decade (Boyle et al., 2011). The three main categories of behavioural symptoms in ASD include impaired social interaction, communication deficits, and restricted/repetitive interests and behaviours (American Psychiatry Association, 1994). Other symptoms associated with autism include sensitivity to sensory stimuli, hyperactivity, resistance to change, cognitive deficits, and seizures (Kootz, Marinelli, & Cohen, 1982; Markram, Rinaldi, & Markram, 2007; Murray, 2010; Sasson, Turner-Brown, Holtzclaw, Lam, & Bodfish, 2008). Although there is a strong multigenetic basis for ASD susceptibility (Bailey et al., 1995; Hallmayer et al., 2011), recent research suggests that environmental, dietary, and gastrointestinal factors may play a role in the etiology and pathogenesis of autism (Ratajczak, 2011; Williams et al., 2011).

Several findings suggest that exposure to propionic acid (PPA), a short-chain fatty acid (SCFA) that is endogenous to the human body, may be associated with ASD. PPA is an intermediary of fatty acid metabolism and is a metabolic end-product of microbial fermentation in the gut (Al-Lahham, Peppelenbosch, Roelofsen, Vonk, & Venema, 2010; Thompson et al., 1990). Parents frequently report an increase in behavioural symptoms when their autistic children ingest refined wheat and dairy products (Jyonouchi, 2009), which contain PPA either as a result of the manufacturing process (e.g., dairy) or as an additive food preservative (e.g., many wheat products) (Brock & Buckel, 2004). Furthermore, consumption of these products can result in

increased production of PPA via bacterial fermentation of undigested food within the gut (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). In support of these anecdotal reports from parents, a randomized controlled trial showed improvement in attention and reduced hyperactivity following implementation of a casein- and gluten-free diet in children with ASD (Whiteley et al., 2010). A subset of autistic children with co-morbid gastrointestinal symptoms have abnormal gut microflora, including elevated levels of *Clostridium* and *Desulfovibrio*, both of which are known to produce SCFAs such as PPA (Finegold et al., 2002; Finegold, 2011; Parracho, Bingham, Gibson, & McCartney, 2005). Exposure to valproic acid early in development, which can increase levels of PPA and other SCFAs, increases the likelihood of ASD (Ornoy, 2009). Furthermore, serum analysis in ASD patients has shown metabolic impairment of glutathione, carnitine, and fatty acids consistent with the physiological effects of PPA (Bell et al., 2004; Filipek, Juranek, Nguyen, Cummings, & Gargus, 2004; James et al., 2006).

PPA is a weak organic acid, and can readily cross the gut-blood barrier and gain access to the central nervous system (CNS) either passively across the blood-brain barrier or via monocarboxylate transporters (Bergersen, Rafiki, & Ottersen, 2002). PPA has widespread effects in the CNS including inhibition of Na^+/K^+ ATPase, increased NMDA receptor sensitivity, alteration of mitochondrial and fatty acid metabolism, immune activation, and changes in gene expression (Brass & Beyerinck, 1988; de Mattos-Dutra et al., 2000; Parab, Nankova, & La Gamma, 2007; Wajner, Latini, Wyse, & Dutra-Filho, 2004; Wyse et al., 1998). In addition, PPA can accumulate within cells, resulting in intracellular acidification which can alter neurotransmitter release, inhibit

gap junctions, and promote intracellular calcium release, all of which can potentially affect neuronal communication and behaviour (Remblier, Pontcharraud, Tallineau, Piriou, & Huguet, 1999; Rorig, Klausa, & Sutor, 1996).

Our laboratory has proposed that alterations in PPA levels or metabolism might be related to ASD and that administration of PPA in rodents may be a useful animal model in which to further our understanding of the biological mechanisms underlying ASD (MacFabe et al., 2007). Repeated, central infusions of PPA in adult rats induced hyperactivity, repetitive behaviours, kindled seizures, social impairments, cognitive deficits, altered brain phospholipid profiles, increased oxidative stress, and an innate neuroinflammatory response (MacFabe et al., 2007, 2008; Nie et al., 2011; Shultz et al., 2008, 2009; Thomas et al., 2010), consistent with findings from ASD patients (Bauman & Kemper, 2005; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005; Wiest, German, Harvey, Watkins, & Hertz-Picciotto, 2009). More recent work in our laboratory investigating the systemic and developmental effects of PPA in adolescent and neonatal rats has been consistent with the symptoms seen in autistic patients and provides further validity to the PPA rodent model of autism (for review of these results see Foley, Tichenoff, Ossenkopp, & MacFabe, 2010; MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011; Shams, Kavaliers, Foley, Ossenkopp, & MacFabe, 2009).

The current study aimed to extend previous findings of this animal model by examining the central effects of two doses of PPA on locomotor and repetitive behaviours in adult rats using the hole-board task. It was expected that PPA treatment would produce increased locomotor and repetitive behaviour in a dose-dependent

manner, similar to previous findings associated with PPA and consistent with symptoms of ASD.

2.2 Method

2.2.1 Subjects

Thirty-five naive male Long-Evans rats were used, weighing 200-225 g (approximately 47-49 days old) at the time of arrival to the facility. Animals were housed individually in standard rat polypropylene cages (W 26 x L 48 x H 21 cm) with *ad libitum* access to food (LabDiet RMH 3000) and tap water in a temperature-controlled colony room (21 ± °C) on a 12:12 h light-dark cycle (lights on at 07:00 h). Behavioural testing occurred during the light phase of the cycle. Animals were left undisturbed for one week prior to the cannulation surgery. All procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the University of Western Ontario Animal Use Subcommittee.

2.2.2 Apparatus

Locomotor activity was monitored using three Versamax Animal Activity Monitors (AccuScan Model DCM-8, Columbus, OH, USA). Each monitor consisted of a clear Plexiglas open field chamber (W 40 cm x L 40 cm x H 30.5 cm) and a clear plastic lid with air holes. Movement was recorded via a grid of infrared beams located on all four sides of the chamber for horizontal activity (16 equally spaced beams 2.54 cm apart and 4.5 cm from the floor) and a grid of infrared beams located on two sides of the chamber for vertical activity (16 beams located 15 cm above the box floor). The automated activity monitors were equipped with a hole-board on the floor of the chamber to measure nose poke responses (see Figure 2.1A-D). The hole-board is an

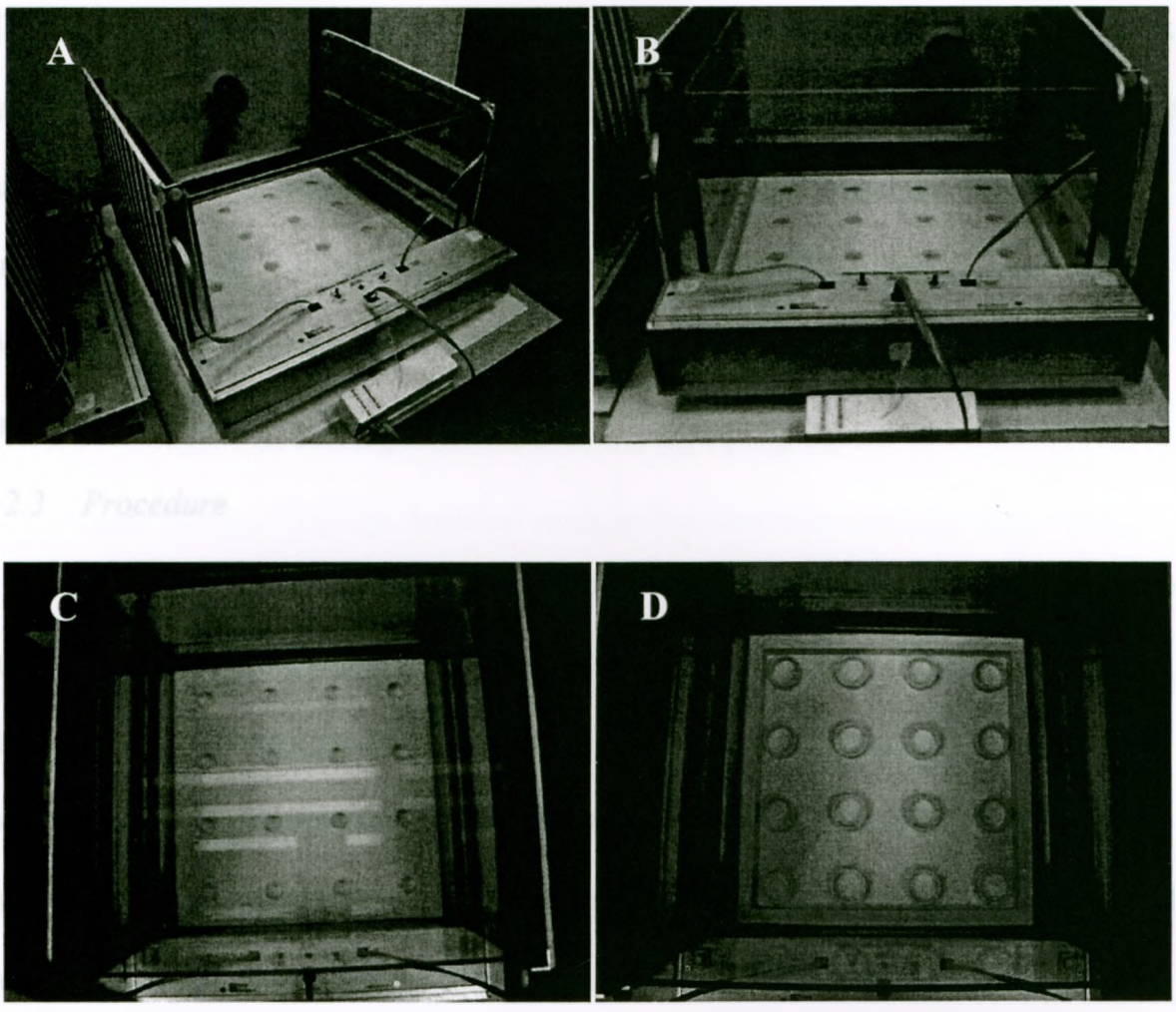


Figure 2.1. Photographs of the automated activity monitors equipped with a hole-board on the floor of the chamber. (A) Side view; (B) Front view; (C) Top-down view; (D) Top-down view with platform removed to show the 16 equally spaced wells beneath the floor of the chamber.

elevated platform with 16 equally spaced holes (2.54 cm diameter) with small wells (5.08 cm diameter) underneath each hole. A set of infrared beam sensors, separate from those recording locomotor activity, are located between the well and the platform, allowing for nose poke counts for each hole to be recorded via beam breaks. VersaMax Analyzer software (Accuscan Model VSA-16, Columbus, OH) recorded data from each automated activity monitor and relayed it to a computer that stored the data for subsequent analysis. All sessions within the automated activity monitors were video-recorded and later reviewed to ensure accuracy of the computer generated nose poke data.

2.2.3 Procedure

2.2.3.1 Surgery. To induce anaesthesia, animals were placed into a sealed plastic box into which 4% isoflurane at 2 L/min oxygen was introduced. The animal was then placed into a Kopf stereotaxic device equipped with a gas flow mask delivering 2.5% isoflurane at 500 mL/min of oxygen to maintain anaesthesia during surgery. Under aseptic conditions, right lateral ventricular cannulation surgery was performed (AP -1.4 mm, ML -1.8 mm, DV -3.0 mm). Each animal was implanted with a 23-gauge guide cannula with the tip in the right lateral ventricle in accordance with a standard rat atlas (Paxinos & Watson, 1998). The indwelling cannula was secured chronically using dental acrylic anchored in place with small stainless steel screws inserted into the skull. A removable obturator sealed the guide cannula and was only removed for infusions during the experiment. Animals received a subcutaneous injection of analgesic (ketoprofen, 1 mL/kg) immediately post-operatively. After surgery, animals were kept

warm under a heating lamp until righting responses and locomotion returned. Animals were housed individually and allowed two weeks recovery prior to testing.

2.2.3.2 Treatment groups and infusion procedure. Following recovery, animals were randomly assigned to one of three groups: high-dose PPA (0.26 M, 4.0 μL, n = 11), low-dose PPA (0.052 M, 4.0 μL, n = 9), or phosphate buffered saline vehicle (PBS, 4.0 μL, n = 15). Propionic acid was dissolved in PBS vehicle, and all solutions were buffered to pH 7.5 using concentrated HCL or NaOH. Each animal received intracerebroventricular (ICV) infusions twice daily (separated by 4 hours) for seven consecutive days. The first infusion occurred during the light phase at 09:00 h. Solutions were infused using a 30 gauge injection cannula that was connected to a Sage syringe pump with sterile PE10 tubing. The tip of the injection cannula protruded 0.5 mm beyond the tip of the guide cannula. The syringe pump dispensed 4.0 μL of solution over a 60 s interval, and the injection cannula remained in place for an additional 60 s before being removed.

2.2.3.3 Testing. Following two weeks of recovery, rats were handled and habituated to the automated activity monitors for two days (30 minutes per day). On the third day, baseline levels of activity and nose poke responses were recorded in the absence of infusion. During the seven treatment days, animals were placed in the automated monitors following the second infusion of the day for 30 minutes to record locomotor activity and nose poke counts (six 5 minute time bins). All wells within the hole-board platform remained empty for the entire duration of behavioural testing (including habituation, baseline, and testing days). Rats were weighed daily to monitor health.

2.2.3.4 Euthanasia. The day after behavioural testing was completed, rats were euthanized via either intraperitoneal injection (euthanyl 270 g/mL, ~0.5 mL per animal) or decapitation. Those euthanized by injection were transcardially perfused with a PBS/4% paraformaldehyde solution. The fixed brains were removed and kept in a sucrose solution for localization of the indwelling cannula. Animals that were decapitated were later used for a lipid analysis study. Brains were quickly removed following decapitation, frozen on dry ice, and stored in a -70 °C freezer until subsequent lipid analysis. Whole blood collected from the trunk was spun in a centrifuge at 3000 x g for five minutes and plasma was collected for lipid analysis.

2.2.4 Behavioural Measures

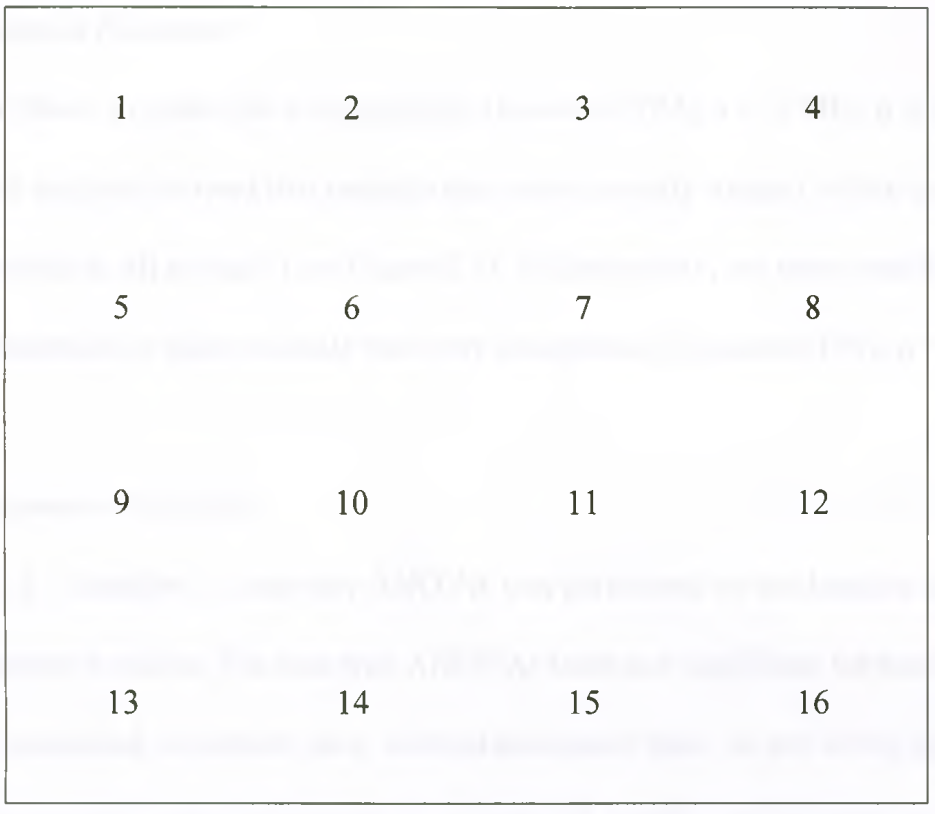
Locomotor activity was analyzed using eight distinct measures. The horizontal activity measures analyzed were: total distance – total horizontal distance (cm) travelled; horizontal movement time – amount of time (s) an animal was engaged in horizontal movement; and number of horizontal movements – the number of horizontal movements separated by a 1 s stop time. The vertical activity measures analyzed were: vertical movement time – amount of time (s) an animal spent in a vertical position; and number of vertical movements – number of vertical movements separated by a 1 s stop time. The repetitive locomotor measures were: clockwise revolutions – the numbers of times an animal moved around in a clockwise circle of at least 2 inches in diameter; counterclockwise revolutions – the number of times an animal moved around in a counterclockwise circle of at least 2 inches in diameter; and the number of stereotypic movements – repeated breaking of the same infrared beam separated by 1 sec or more.

Nose poke behaviour was analyzed using total nose poke counts and hole category. Total nose poke counts are the total number of nose pokes across an entire testing session (includes all 16 different holes in the hole-board). Hole category data classified the holes into three categories based on location within the open-field apparatus in order to determine if there was a pattern of hole preference. The three hole categories based on location included corner holes (1, 4, 13, and 16); centre holes (6, 7, 10, and 11), and wall holes (2, 3, 5, 8, 9, 12, 14, and 15) (see Figure 2.2). In order to control for differences in total nose pokes, each hole category was calculated as a percent of total nose pokes for each rat (e.g., centre hole nose pokes divided by total number of nose pokes multiplied by 100).

2.2.5 Statistical Analysis

Data were analyzed for main effects and interactions using a repeated measures split-plot analysis of variance (ANOVA) with drug treatment (PBS, low-dose PPA, and high-dose PPA) as the between-subjects factor and infusion day (7 infusions days) and time block (six 5 minute time blocks) as the within subjects factors (with the exception of hole category, which was analyzed with only infusion day as the within subjects factor). The dependent variables were several locomotor variables and two nose poke response variables. Separate statistical analyses were conducted for each variable. A one-way ANOVA was conducted on the baseline data to ensure that there were no individual differences prior to treatment days. For any variable in which the baseline ANOVA indicated significant group differences, a repeated measures ANCOVA was performed, using the baseline data as a co-variate. Where appropriate, post-hoc pair-

Back wall of chamber



Front wall of chamber

Figure 2.2. Schematic of the hole-board numbering system in the automated activity monitor, indicating four corner holes (1, 4, 13, and 16), four centre holes (6, 7, 10, and 11), and eight wall holes (2, 3, 5, 8, 9, 12, 14, and 15).

wise comparisons were conducted using Tukey's HSD or Sidak. Significance was set to $\alpha = .05$.

2.3 Results

2.3.1 Cannula Placement

For those animals that were perfused (low-dose PPA, n = 9; PBS, n = 3), histological analysis showed that cannula tips were correctly located within the right lateral ventricle in all animals (see Figure 2.3). Unfortunately, we were unable to verify cannula placement in those animals that were decapitated (high-dose PPA, n = 11; PBS n = 12).

2.3.2 Locomotor Variables

2.3.2.1 Baseline. A one-way ANOVA was performed on the baseline data for each locomotor variable. The one-way ANOVAs were not significant for total distance travelled, horizontal movement time, vertical movement time, or any of the repetitive activity measures (number of stereotypic movements, number of clockwise revolutions, and number of counterclockwise revolutions). Thus, there were no differences in locomotor activity among the treatment groups for these behavioural measures prior to the first infusion day. The one-way ANOVA was significant on baseline day for number of horizontal movements and number of vertical movements; therefore, baseline data were used as a covariate on infusion days for these variables.

2.3.2.2 Horizontal activity measures. Analysis revealed a significant day x treatment interaction for number of horizontal movements (ANCOVA), $F(12, 186) = 3.25$, $p < .01$, and horizontal movement time (ANOVA), $F(12, 192) = 2.74$, $p < .05$. There was a significant main effect of treatment for total distance traveled (ANOVA),

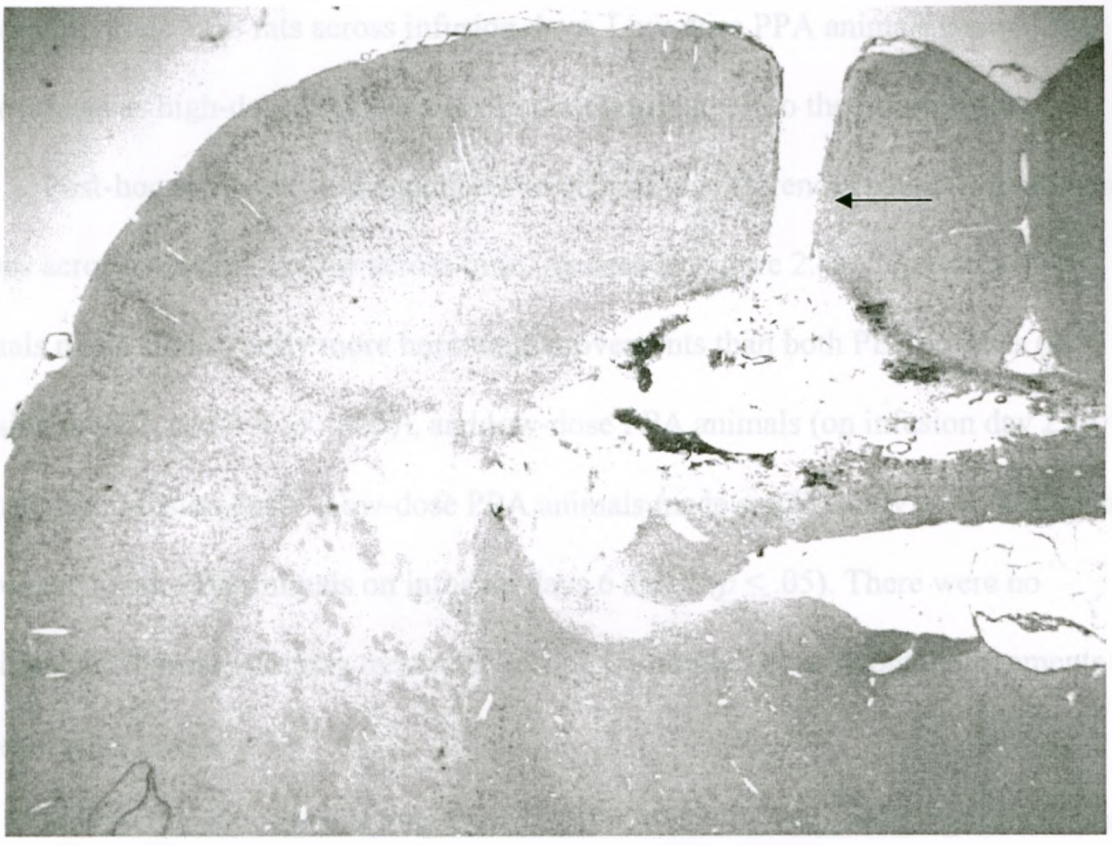


Figure 2.3. Representative photomicrograph showing proper cannula placement (arrow) into the right lateral ventricle using an H&E stain

$F(2, 32) = 16.67$, $p < .001$. In general, PPA infusions produced an increase in all three horizontal activity measures that was dose dependent. High-dose PPA infused rats travelled further, made more horizontal movements, and spent more time traveling horizontally than PBS rats across infusion days. Low-dose PPA animals showed the same effects as high-dose PPA animals, but not until later into the infusion schedule.

Post-hoc analyses were conducted to determine differences between treatment groups across infusion days or across time. As seen in Figure 2.4A, high-dose PPA animals made significantly more horizontal movements than both PBS animals (on infusion days 2, and 4-7 ($p < .05$)), and low-dose PPA animals (on infusion day 2 ($p < .05$)) across infusion days. Low-dose PPA animals made significantly more horizontal movements than PBS animals on infusion days 6 and 7 ($p < .05$). There were no significant differences among treatment groups for number of horizontal movements on infusion days 1 and 3.

Across the 30 minute testing sessions, the ANCOVA showed a significant time x treatment interaction for number of horizontal movements, $F(10, 155) = 8.35$, $p < .001$. Post-hoc analyses were conducted across the six 5 minute time bins for number of horizontal movements. On infusion day 1, there were no significant differences among groups during the entire 30 minute testing session (see Figure 2.4B). On infusion day 4, low-dose PPA animals made significantly less horizontal movements than PBS animals during the first 5 minutes ($p < .05$), and high-dose PPA animals made significantly more horizontal movements than PBS animals between 20 and 25 minutes ($p < .05$; see Figure 2.4C). There were no significant differences among treatment groups for all other times during the testing session. On infusion day 7, both PPA groups made significantly more

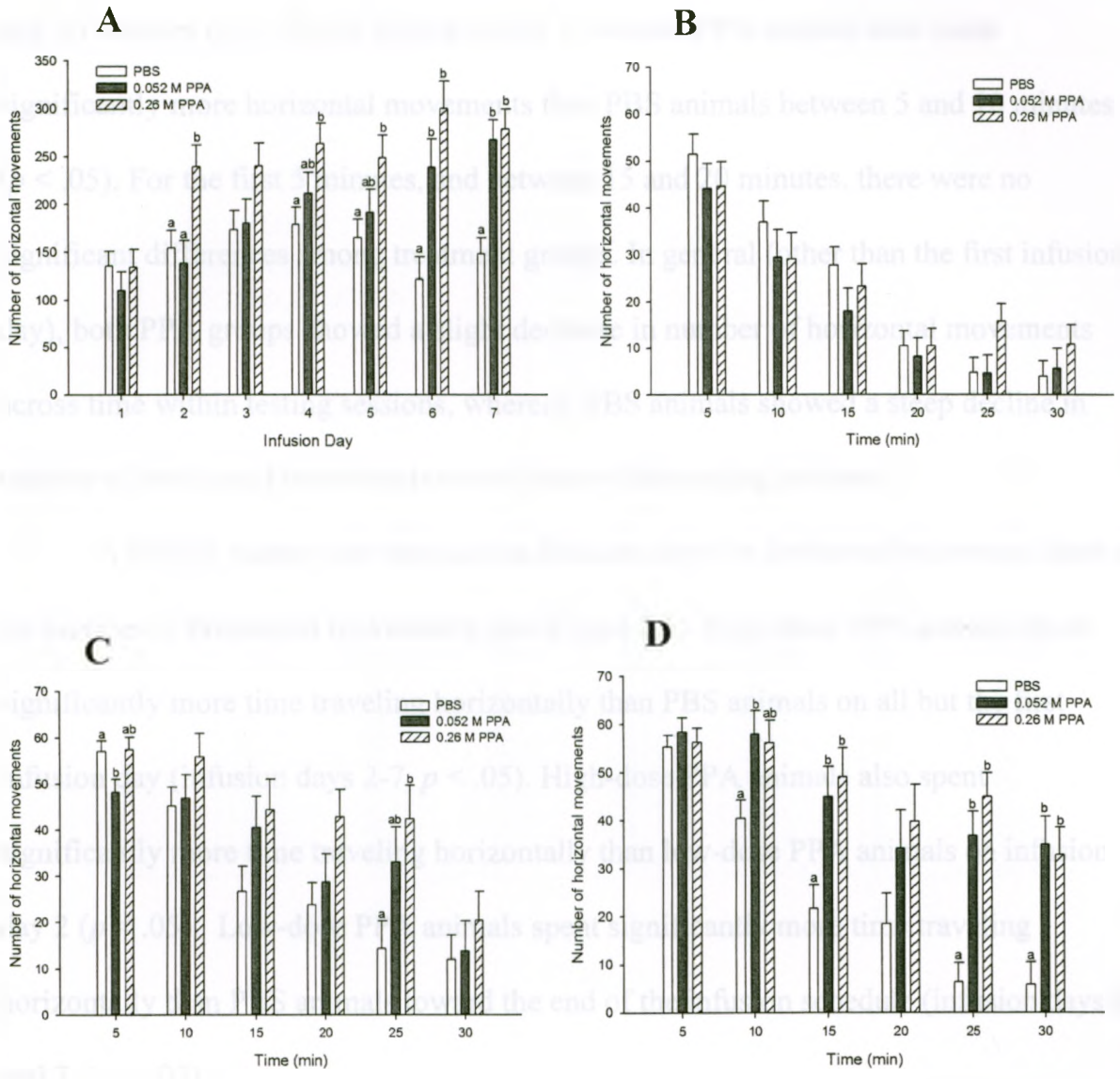


Figure 2.4. Mean number of horizontal movements + *SEM* across infusion days (A) or across time (B. infusion day 1; C. infusion day 4; D. infusion day 7) in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day or that particular time ($p < .05$; no superscripts indicate no significant difference between groups).

horizontal movements than PBS animals between 10 and 15 minutes and between 20 and 30 minutes ($p < .05$; see Figure 2.4D). Low-dose PPA animals also made significantly more horizontal movements than PBS animals between 5 and 10 minutes ($p < .05$). For the first 5 minutes, and between 15 and 20 minutes, there were no significant differences among treatment groups. In general (other than the first infusion day), both PPA groups showed a slight decrease in number of horizontal movements across time within testing sessions, whereas, PBS animals showed a steep decline in number of horizontal movements across time within testing sessions.

A similar pattern was seen across infusion days for horizontal movement time as for number of horizontal movements (see Figure 2.5). High-dose PPA animals spent significantly more time traveling horizontally than PBS animals on all but the first infusion day (infusion days 2-7, $p < .05$). High-dose PPA animals also spent significantly more time traveling horizontally than low-dose PPA animals on infusion day 2 ($p < .05$). Low-dose PPA animals spent significantly more time traveling horizontally than PBS animals toward the end of the infusion schedule (infusion days 6 and 7, ($p < .05$).

Post-hoc analyses for total distance traveled across infusion days were similar to number of horizontal movements and horizontal movement time. High-dose PPA animals traveled significantly more than PBS animals on infusion days 2-7 ($p < .05$; see Figure 2.6). High-dose PPA animals also traveled significantly more than low-dose PPA animals on infusion day 3 ($p < .05$). Low-dose PPA animals traveled significantly more than PBS animals on infusion days 4, 6, and 7 ($p < .05$). There were no significant differences among treatment groups for total distance traveled on infusion day 1.

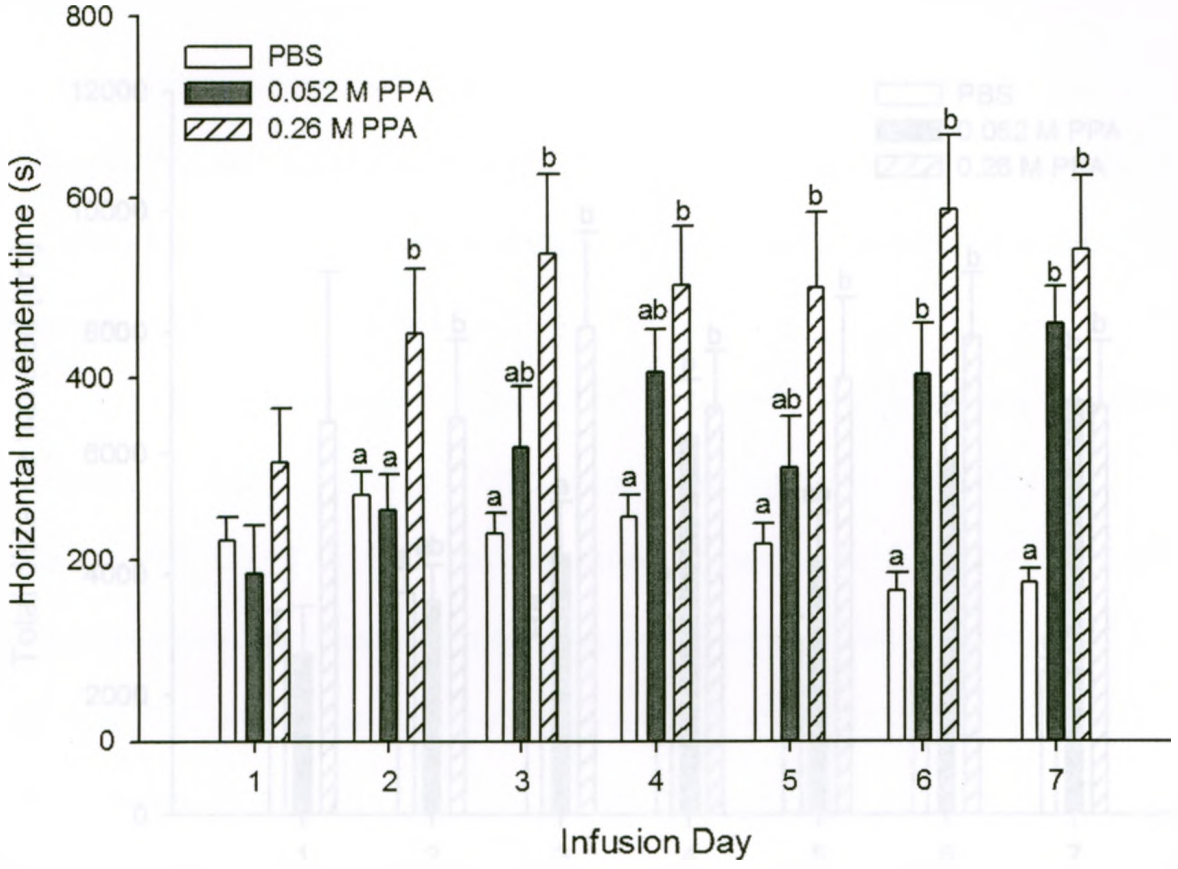


Figure 2.5. Mean horizontal movement time (s) + *SEM* across infusion days in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

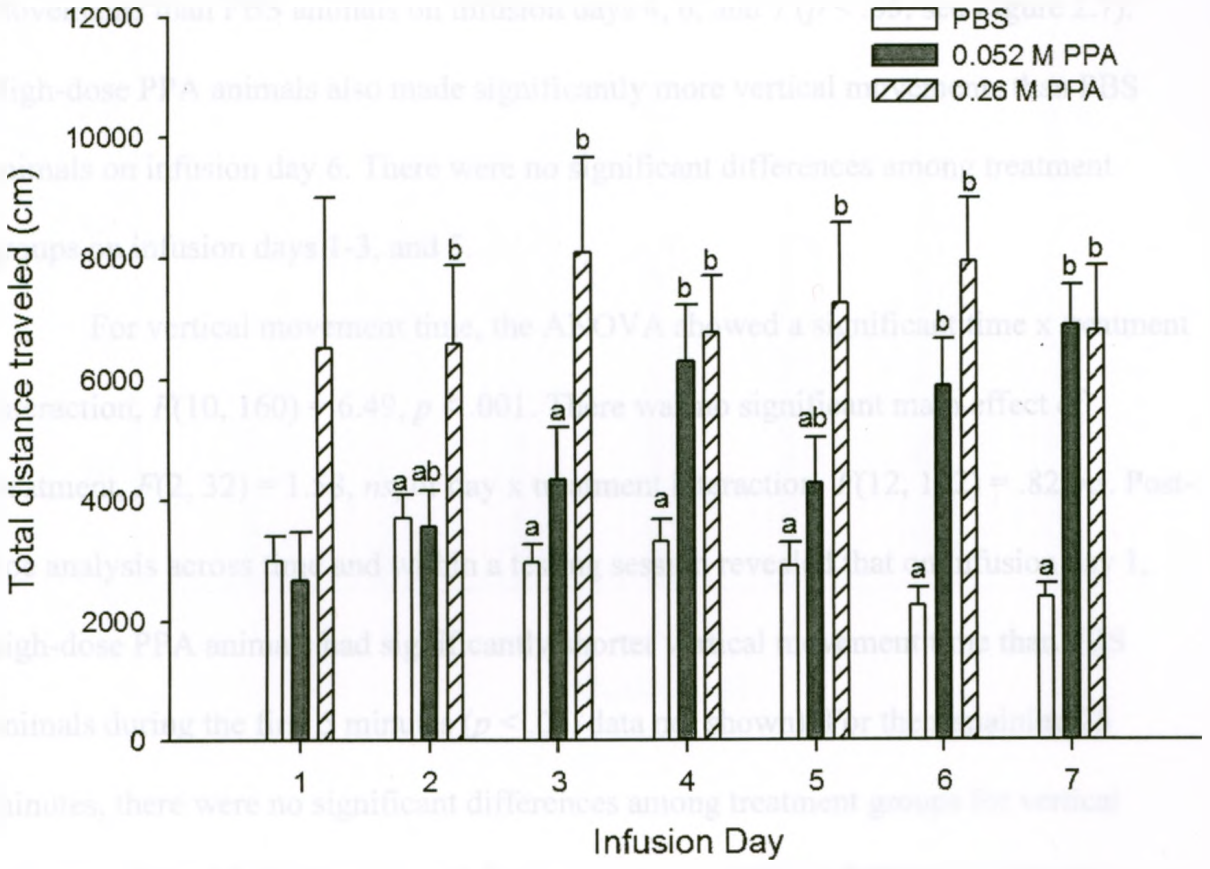


Figure 2.6. Mean total distance traveled (cm) + *SEM* across infusion days in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

2.3.2.3 Vertical activity measures. The ANCOVA revealed a significant day x treatment interaction for number of vertical movements, $F(12, 186) = 2.40$, $p < .05$, but no significant time x treatment interaction, $F(10, 155) = .27$, *ns*. Post-hoc analysis across infusion days showed that low-dose PPA animals made significantly more vertical movements than PBS animals on infusion days 4, 6, and 7 ($p < .05$; see Figure 2.7). High-dose PPA animals also made significantly more vertical movements than PBS animals on infusion day 6. There were no significant differences among treatment groups on infusion days 1-3, and 5.

For vertical movement time, the ANOVA showed a significant time x treatment interaction, $F(10, 160) = 6.49$, $p < .001$. There was no significant main effect of treatment, $F(2, 32) = 1.58$, *ns*, or day x treatment interaction, $F(12, 192) = .82$, *ns*. Post-hoc analysis across time and within a testing session revealed that on infusion day 1, high-dose PPA animals had significantly shorter vertical movement time than PBS animals during the first 5 minutes ($p < .05$, data not shown). For the remaining 25 minutes, there were no significant differences among treatment groups for vertical movement time. On infusion day 4, there were no significant differences among treatment groups for vertical movement time during the entire 30 minute testing session (data not shown). On infusion day 7, there were no significant differences among treatment groups for vertical movement time for the first 20 minutes of the testing session. During the remainder of the testing session, low-dose PPA animals had significantly longer vertical movement time than PBS (time 20-30, $p < .05$) and high-dose PPA animals (time 25-30, $p < .05$, data not shown).

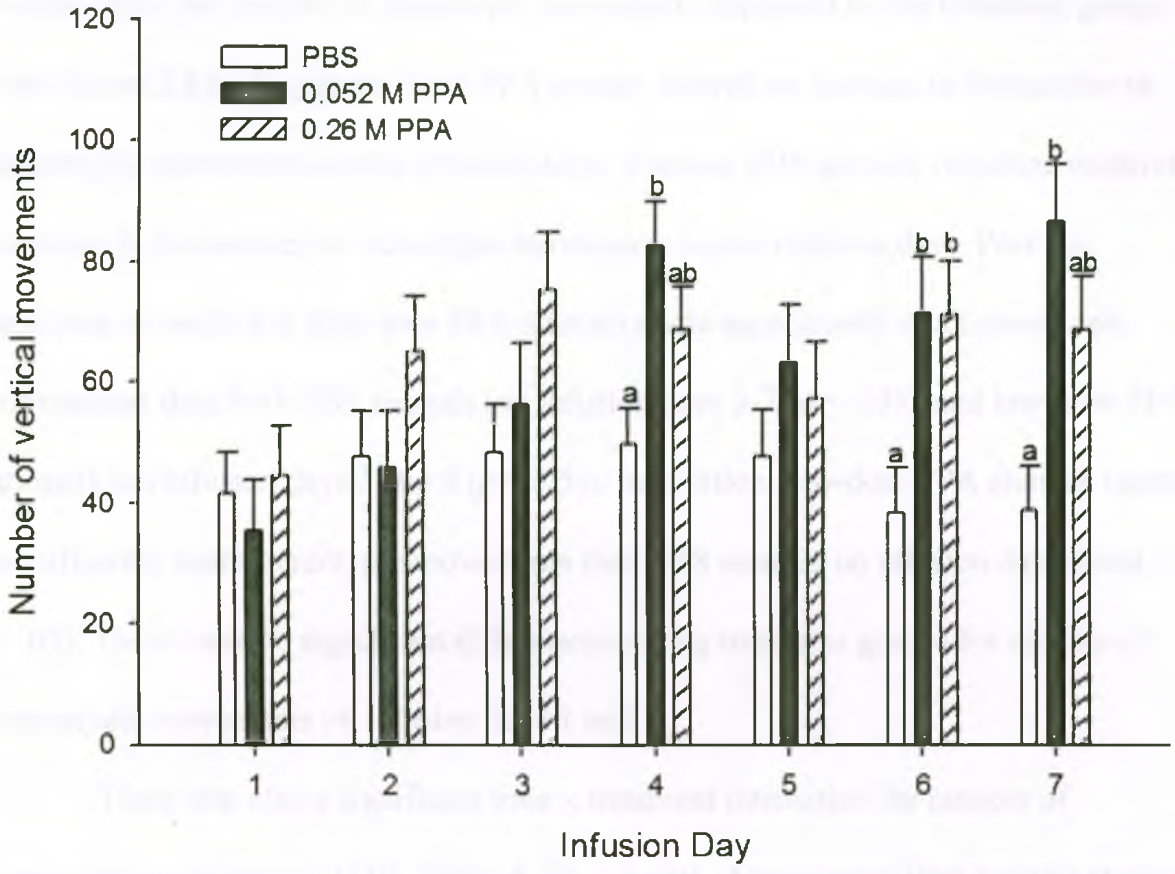


Figure 2.7. Mean number of vertical movements + *SEM* across infusion days in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

2.3.2.4 Repetitive activity measures. The ANOVA revealed a significant day x treatment interaction for number of stereotypic movements, $F(12, 192) = 3.15$, $p < .01$. Across days, the number of stereotypic movements depended on the treatment groups (see Figure 2.8A). In general, both PPA groups showed an increase in the number of stereotypic movements across infusion days; whereas, PBS animals remained relatively constant in the number of stereotypic movements across infusion days. Post-hoc analyses revealed that high-dose PPA animals made significantly more stereotypic movements than both PBS animals (on infusion days 3-7 ($p < .05$)), and low-dose PPA animals (on infusion days 3 and 6 ($p < .05$)). In addition, low-dose PPA animals made significantly more stereotypic movements than PBS animals on infusion days 6 and 7 ($p < .05$). There were no significant differences among treatment groups for number of stereotypic movements on infusion days 1 and 2.

There was also a significant time x treatment interaction for number of stereotypic movements, $F(10, 160) = 8.30$, $p < .001$. Across time, PBS animals showed a decrease in the number of stereotypic movements. Both PPA groups showed a decrease in the number of stereotypic movements across time, but much more gradually than PBS animals. Post-hoc analysis for infusion day 1 showed that low-dose PPA animals made significantly fewer stereotypic movements than both PBS and high-dose PPA animals between 15 and 20 minutes ($p < .05$; see Figure 2.8B). There were no significant differences among treatment groups for number of stereotypic movements during the remainder of the testing session. On infusion day 4, high-dose PPA animals made significantly more stereotypic movements than PBS animals during the first 5 minutes ($p < .05$) and between 20 and 25 minutes ($p < .05$; see Figure 2.8C). There were

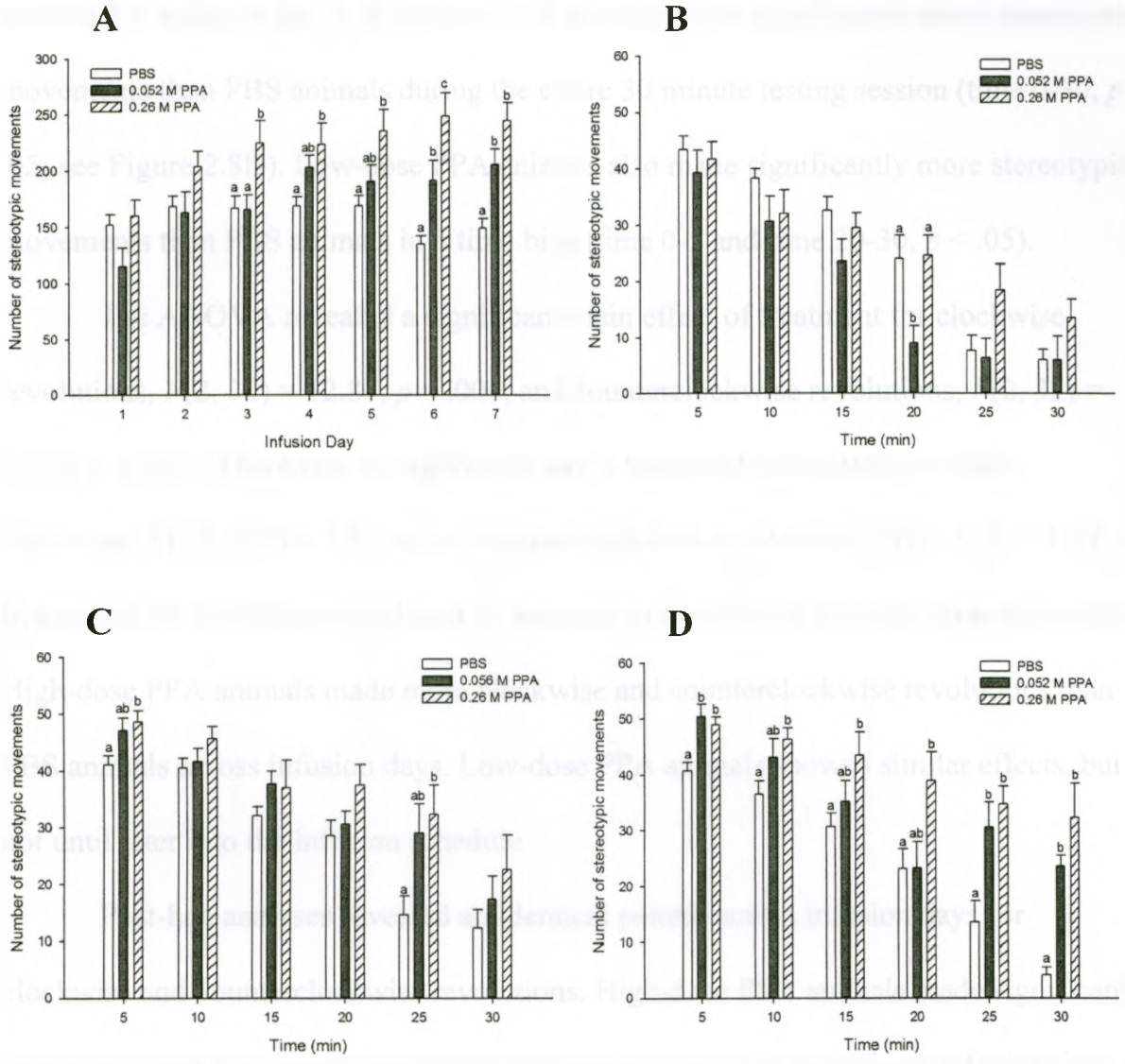


Figure 2.8. Mean number of stereotypic movements + *SEM* across infusion days (A) or across time (B. infusion day 1; C. infusion day 4; D. infusion day 7) in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a, b, or c) indicate a significant difference between treatment groups on that particular infusion day or that particular time ($p < .05$; no superscripts indicate no significant difference between groups).

no significant differences among treatment groups at any other times during the testing session. On infusion day 7, high-dose PPA animals made significantly more stereotypic movements than PBS animals during the entire 30 minute testing session (time 0-30, $p < .05$; see Figure 2.8D). Low-dose PPA animals also made significantly more stereotypic movements than PBS animals in 3 time bins (time 0-5 and time 20-30, $p < .05$).

The ANOVA revealed a significant main effect of treatment for clockwise revolutions, $F(2, 32) = 12.87$, $p < .001$, and counterclockwise revolutions, $F(2, 32) = 12.15$, $p < .001$. There was no significant day x treatment interaction for either clockwise, $F(12, 192) = 1.87$, *ns*, or counterclockwise revolutions, $F(12, 192) = 1.97$, *ns*. In general, PPA infusions produced an increase in revolutions that was dose dependant. High-dose PPA animals made more clockwise and counterclockwise revolutions than PBS animals across infusion days. Low-dose PPA animals showed similar effects, but not until later into the infusion schedule.

Post-hoc analyses revealed an identical pattern across infusion days for clockwise and counterclockwise revolutions. High-dose PPA animals made significantly more clockwise and counterclockwise revolutions than PBS animals on infusion days 3 to 7 ($p < .05$; see Figure 2.9 for clockwise revolutions). Low-dose PPA animals made significantly more clockwise and counterclockwise revolutions than PBS animals on infusion days 6 and 7 ($p < .05$). There were no significant differences among treatment groups for clockwise or counterclockwise revolutions on infusion day 1 or 2.

Across the 30 minute testing session, the ANOVA showed a significant time x treatment interaction for clockwise revolutions, $F(10, 160) = 2.87$, $p < .05$, but not for counterclockwise revolutions, $F(10, 160) = 1.81$, *ns*. In general, PBS animals showed a

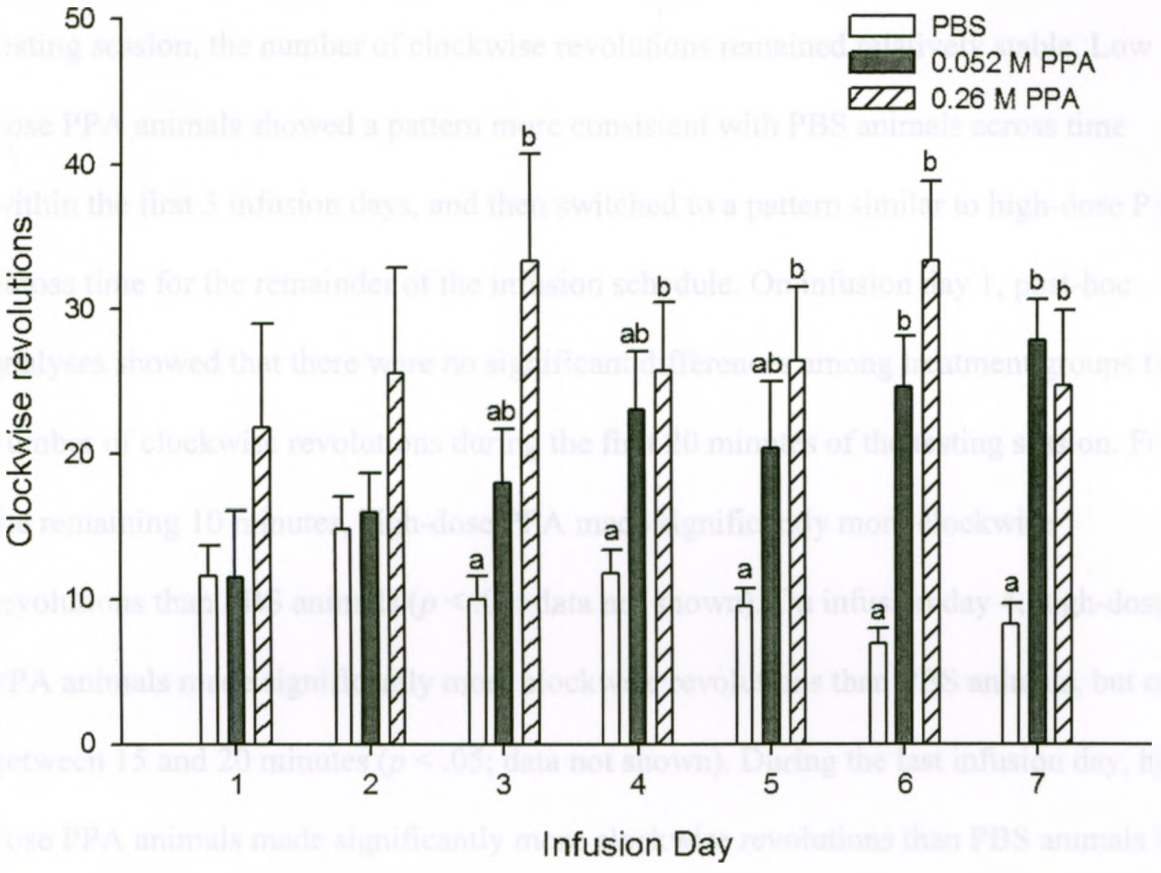


Figure 2.9. Mean number of clockwise revolutions + *SEM* across infusion days in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

sharp decline in number of clockwise revolutions across time within a testing session. In contrast, high-dose PPA animals showed a more gradual decline in clockwise revolutions within the first half of the testing session, and during the second half of the testing session, the number of clockwise revolutions remained relatively stable. Low-dose PPA animals showed a pattern more consistent with PBS animals across time within the first 3 infusion days, and then switched to a pattern similar to high-dose PPA across time for the remainder of the infusion schedule. On infusion day 1, post-hoc analyses showed that there were no significant differences among treatment groups for number of clockwise revolutions during the first 20 minutes of the testing session. For the remaining 10 minutes, high-dose PPA made significantly more clockwise revolutions than PBS animals ($p < .05$; data not shown). On infusion day 4, high-dose PPA animals made significantly more clockwise revolutions than PBS animals, but only between 15 and 20 minutes ($p < .05$; data not shown). During the last infusion day, high-dose PPA animals made significantly more clockwise revolutions than PBS animals for 2 time bins (time 5-10 and time 15-20, $p < .05$; data not shown). Low-dose PPA animals made significantly more clockwise revolutions than PBS animals during the first 5 minutes ($p < .05$) and the last 15 minutes ($p < .05$).

2.3.3 Nose Poke Variables

2.3.3.1 Nose poke counts. The one-way ANOVA performed on the baseline data for total nose poke counts revealed no significant group differences. Therefore, there were no significant differences among treatment groups in number of nose pokes prior to the first infusion day.

The ANOVA revealed a significant day x treatment interaction across infusion days for number of nose pokes, $F(12, 192) = 2.96$, $p < .01$. As seen in Figure 2.10A, PPA infusions resulted in a dose-dependent increase in number of nose pokes toward the end of the infusion schedule. Post-hoc analyses showed that high-dose PPA animals made significantly more nose pokes than PBS animals on infusion days 5 and 6 ($p < .05$). On infusion day 7, low-dose PPA animals made significantly more nose pokes than PBS animals ($p < .05$). There were no significant differences among treatment groups for number of nose pokes on infusion days 1 through 4.

There was a significant time x treatment interaction for number of nose pokes, $F(10, 160) = 5.11$, $p < .01$. Across time within a testing session, PBS animals showed a steep decrease in number of nose pokes. In contrast, high-dose PPA animals showed a slower decrease in number of nose pokes across time. Low-dose PPA animals resembled PBS animals across time for most of the infusion days, with the exception of infusion days 6 and 7, where low-dose PPA animals showed a pattern more similar to high-dose PPA animals across time. Post-hoc analysis on infusion day 1 showed that there were no significant differences among treatment groups for number of nose pokes during the first 20 minutes of the testing session (see Figure 2.10B). During the last 10 minutes, high-dose PPA animals made significantly more nose pokes than PBS animals ($p < .05$). On infusion day 4, high-dose PPA animals made significantly more nose pokes than PBS animals during 2 time bins (time 10-15 and time 20-25, $p < .05$; see Figure 2.10C). During the last infusion day, high-dose PPA animals made significantly more nose pokes

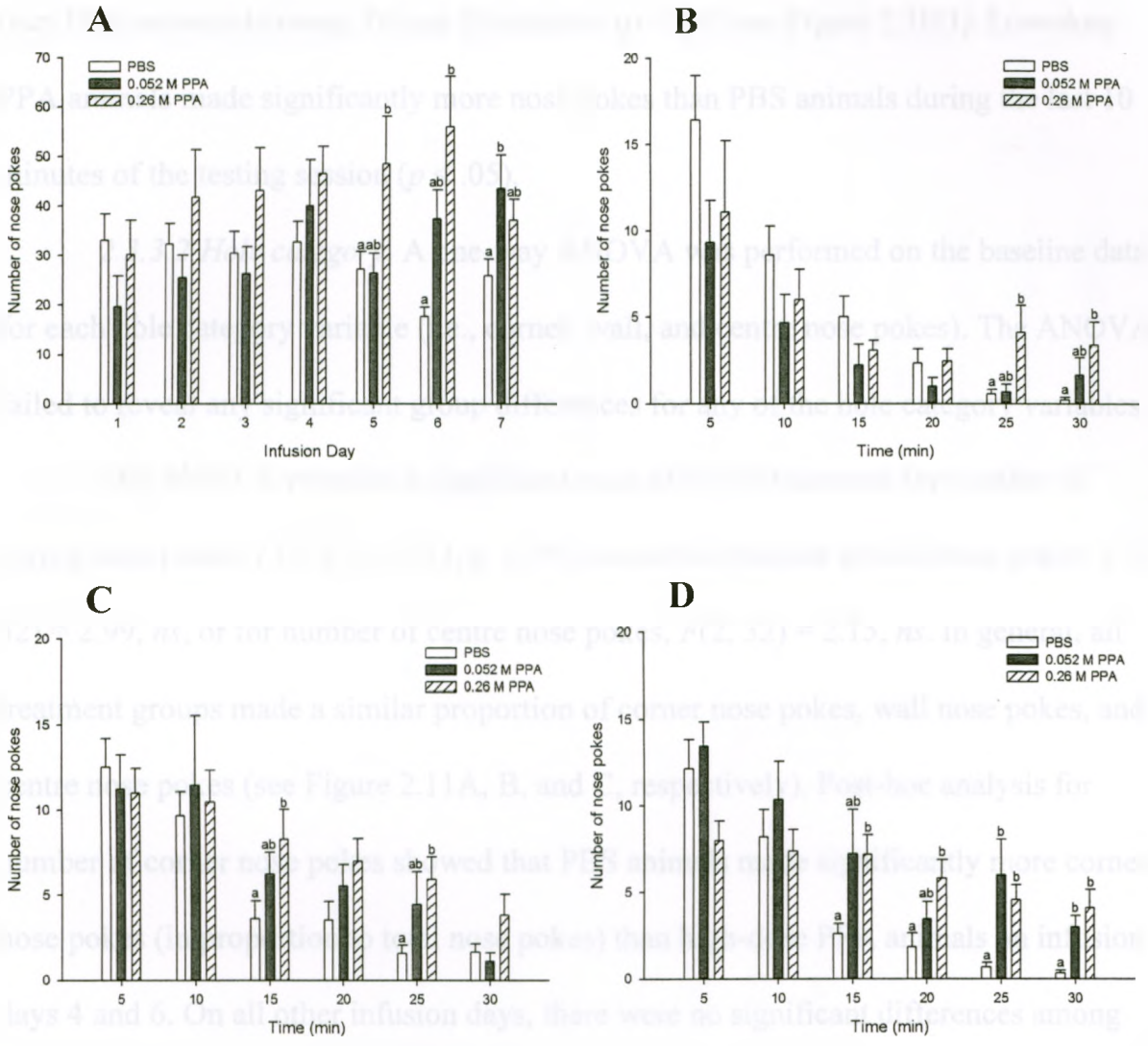


Figure 2.10. Mean number of nose pokes + *SEM* across infusion days (A) or across time (B. infusion day 1; C. infusion day 4; D. infusion day 7) in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day or that particular time ($p < .05$; no superscripts indicate no significant difference between groups).

than PBS animals between 10 and 30 minutes ($p < .05$; see Figure 2.10D). Low-dose PPA animals made significantly more nose pokes than PBS animals during the last 10 minutes of the testing session ($p < .05$).

2.3.3.2 Hole category. A one-way ANOVA was performed on the baseline data for each hole category variable (i.e., corner, wall, and centre nose pokes). The ANOVAs failed to reveal any significant group differences for any of the hole category variables.

The ANOVA revealed a significant main effect of treatment for number of corner nose pokes, $F(2, 32) = 4.13$, $p < .05$, but not for number of wall nose pokes, $F(2, 32) = 2.99$, *ns*, or for number of centre nose pokes, $F(2, 32) = 2.15$, *ns*. In general, all treatment groups made a similar proportion of corner nose pokes, wall nose pokes, and centre nose pokes (see Figure 2.11A, B, and C, respectively). Post-hoc analysis for number of corner nose pokes showed that PBS animals made significantly more corner nose pokes (in proportion to total nose pokes) than high-dose PPA animals on infusion days 4 and 6. On all other infusion days, there were no significant differences among treatment groups for number of corner nose pokes.

2.4 Discussion

To summarize, the results from the present study indicated that centrally infused PPA in adult male rats produced increased locomotor and nose poking behaviour in a dose-dependent manner. In general, high-dose PPA animals were more hyperactive, made more vertical movements, displayed more stereotypic and repetitive movements, and nose poked more often than PBS animals across infusion days and across time. Low-dose PPA animals showed similar effects to high-dose PPA animals, but not until later into the infusion schedule. In particular, low-dose PPA animals showed locomotor

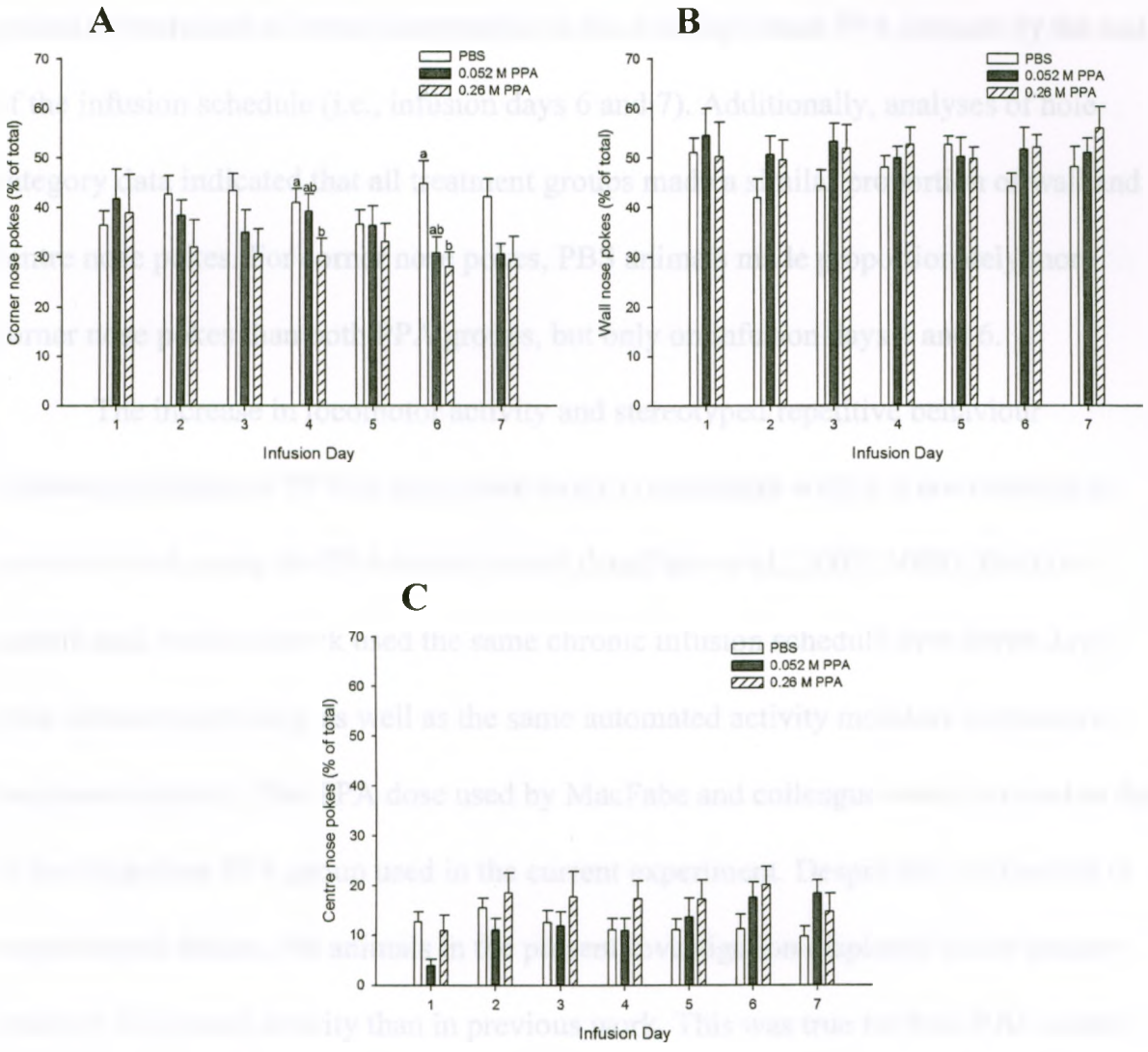


Figure 2.11. Mean number of corner nose pokes (A), wall nose pokes (B), or centre nose pokes (C) expressed as a percent of total nose pokes + *SEM* across infusion days in PBS (n = 15), low-dose PPA (0.052 M, n = 9), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

activity levels similar to those of PBS animals at the start of the infusion schedule, but gradually increased to levels comparable to those of high-dose PPA animals by the end of the infusion schedule (i.e., infusion days 6 and 7). Additionally, analyses of hole category data indicated that all treatment groups made a similar proportion of wall and centre nose pokes. For corner nose pokes, PBS animals made proportionately more corner nose pokes than both PPA groups, but only on infusion days 4 and 6.

The increase in locomotor activity and stereotyped/repetitive behaviour following infusion of PPA in the current study is consistent with, but not identical to, previous work using the PPA rodent model (MacFabe et al., 2007, 2008). Both the current and previous work used the same chronic infusion schedule over seven days (two infusions per day), as well as the same automated activity monitors to measure locomotor activity. The PPA dose used by MacFabe and colleagues was identical to that of the high-dose PPA group used in the current experiment. Despite the similarities in experimental design, the animals in the present investigation displayed much greater levels of horizontal activity than in previous work. This was true for both PBS control animals and high-dose PPA animals. This is particularly notable for total distance traveled. For instance, on infusion day 3, the mean total distance traveled was 2968 cm, 4357 cm, and 8086 cm for PBS, low-dose PPA, and high-dose PPA animals, respectively. In contrast, previous work showed an approximate mean total distance traveled of 1000 cm in PBS animals and 3700 cm in PPA animals on infusion day 3 (MacFabe et al., 2007). Unlike the horizontal movement measures, the vertical movement measures and number of stereotypic movements in PBS and high-dose PPA

animals in the current study were similar to that of earlier work (MacFabe et al., 2008; Thomas et al., 2010)

Given that the present experiment used the same strain and gender of rat (i.e., male Long-Evans) and the same dose and infusion regimen of PPA (i.e., two infusions per day for seven consecutive days), and that PBS animals also showed an increase in horizontal activity measures, it is highly unlikely that the increase was due to a fundamental difference in the ability of these particular rats to metabolize or compensate for PPA infusions, as compared to rats used in previous work using this model. The most plausible explanation for the increase in horizontal activity seen in the current experiment is the addition of the hole-board on the floor of the automated activity monitors. The hole-board was the only difference in experimental design in the current study suggesting that exploration in the hole-board apparatus may influence overall locomotor activity.

Several studies have shown that locomotor activity varies independently of nose poking in rodents (Abel, 1995; Durcan & Lister, 1989; File & Wardill, 1975a, 1975b), but others have found no such dissociation or mixed results depending on drug treatment (Kliethermes & Crabbe, 2006; Moy et al., 2008). Kliethermes and Crabbe showed that although drug treatment resulted in similar effects on both locomotor and nose poking behaviours at a group level, analysis at the individual level in untreated mice revealed that these behaviours could vary independently. Overall, there appears to be a complicated and equivocal relationship between nose poking and locomotion as measured in the hole-board apparatus.

Both PPA groups showed a decrease in locomotor behaviour within a 30 minute testing session. This decrease could reflect PPA metabolism and is consistent with the known 18 to 57 minute half-life of PPA when administered to rats (Brusque et al., 1999). Across infusion days, low-dose PPA animals gradually reached levels of locomotor activity and nose poking comparable to that of high-dose PPA animals. Chronic low doses of PPA eventually led to abnormal motor behaviour, suggesting that compensatory mechanisms were unable to effectively counteract the effects of PPA. These compensatory mechanisms may include increased synthesis of certain enzymes, such as propionyl CoA decarboxylase which metabolizes PPA, or carbonic anhydrase which helps maintain acid-base balance (Nguyen et al., 2007; Schlue, Dorner, Rempe, & Riehl, 1991)

Propionic acid has several physiological/biochemical effects that alter neural function which can account for the increased locomotor behaviour seen in PPA-infused animals. PPA can inhibit Na^+/ K^+ ATPase, increase NMDA receptor sensitivity, promote intracellular calcium release, and elevate nitric oxide, all of which can alter neurotransmission in brain regions relevant to locomotor behaviour (de Mattos-Dutra et al., 2000; Wajner et al., 2004; Wyse et al., 1998).

As a weak organic acid, PPA can passively accumulate in CNS cells resulting in a reduction in intracellular pH, which has many physiological consequences (Karuri, Dobrowsky, & Tannock, 1993). Previous work in our laboratory has shown that PPA and other short chain fatty acids (i.e., butyric acid and sodium acetate) produce similar behavioural impairments, but the non-acidic analogue of PPA, 1-propanol, did not produce behavioural impairments, suggesting that pH dependent mechanisms are an

important component of the observed effects (MacFabe et al., 2007; Shultz et al., 2008, 2009; Thomas et al., 2010). Intracellular acidification of neurons is known to increase the synthesis and release of several neurotransmitters that can influence locomotor activity, including glutamate, dopamine, norepinepherine, and serotonin (Cannizzaro, Monastero, Vacca, & Martire, 2003; Remblier et al., 1999; Severson, Wang, Pieribone, Dohle, & Richerson, 2003). Furthermore, 3-nitropropionic acid, a derivative of PPA, causes motor abnormalities and is used as a rodent model of Huntington's disease, emphasizing that intracellular pH reduction is linked to increased locomotor behaviour (Brouillet, Jacquard, Bizat, & Blum, 2005).

Another consequence of intracellular acidification is a reduction in intercellular coupling via the rapid and reversible closure of gap junctions (Rorig et al., 1996). Gap junctions play a vital role in electrotonic transmission in brain areas involved in locomotor activity, including the basal ganglia, prefrontal cortex, and hippocampal formation (O'Donnell & Grace, 1997; Velazquez, Han, & Carlen, 1997). In addition, intrastriatal infusions of gap junction blockers produce movement stereotypies in rodents (Moore & Grace, 2002). Thus, the increased locomotor behaviour seen in PPA treated rats could be a consequence of the closure of gap junctions.

In addition to the abnormal locomotor behaviour observed in PPA treated animals, there was also an increase in nose poking compared to controls. PPA infusions led to a dose-dependent increase in nose poking toward the end of the infusion schedule. Nose poking, sometimes referred to as head-dipping, has typically been interpreted as representing exploratory behaviour or investigation of a novel environment (File & Wardill, 1975a, 1975b). However, many researchers have argued that nose poking in the

hole-board apparatus does not measure exploratory behaviour, but instead reflects the anxiety state of the animal (Takeda, Tsuji, & Matsumiya, 1998), an escape response (Brown & Nemes, 2008), or stereotyped behaviour (Makanjuola, Hill, Maben, Dow, & Ashcroft, 1977). The widespread opinions regarding what the hole-board task is actually measuring in rodents are likely due, at least in part, to methodological differences. Across studies, the hole-board apparatus often differs in total number of holes (usually 4 or 16), the location of the holes (centrally, peripherally, or equally dispersed within the floor of the apparatus), the diameter of the holes (allowing the rodent to either insert only its nose, or its entire head), the depth of the hole (ranging from 1 cm to 20 cm), and whether the hole-board is enclosed or open (occasionally the hole-board is used as an elevated platform without walls). In addition, the amount of time that rodents are observed within the hole-board apparatus ranges widely (e.g., 5 minutes, 10 minutes, 30 minutes, and 3 hours). The variability of the hole-board apparatus has led to conflicting interpretations of nose poking behaviour, and this underscores the need for a standardized hole-board apparatus. To my knowledge, no study has acknowledged these methodological differences, and in order for the hole-board test to be valid and useful, it is imperative that these issues be addressed in the future.

Another important consideration in the interpretation of the hole-board task is the complexity of exploratory behaviour. When rodents are exposed to a novel environment, they often exhibit exploratory behaviour, such as locomoting around the environment or orienting toward novelty (Ballaz, 2009; Berlyne, 1950). Presumably, exploratory behaviour allows the animal to gather relevant survival-related information about the unfamiliar place, such as food sources, mating opportunities, or the presence

of predators. With continued exposure, the environment becomes familiar, and exploration decreases as animals become habituated. Decreased exploratory behaviour in response to repeated exposure to a novel environment is one of the most common forms of habituation seen in rodents, and habituation is often considered the simplest form of learning (Leussis & Bolivar, 2006). However, there are a variety of factors influencing exploratory behaviour, including arousal level, attention, learning, memory, and fear of novelty (Berlyne, 1969; Bronson, 1968). The presence of these mitigating factors in exploratory behaviour makes the interpretation of any measure of exploration complex. Although levels of nose poking may reflect exploratory behaviour, any increase or decrease in nose poking following treatment may be due to a variety of factors, suggesting that interpretation of results may require complementary information from evaluations of anxiety, learning and memory, etc.

Given the complexity of interpreting nose poking behaviour in the hole-board apparatus, there are several possible explanations for the dose-dependent increase in nose pokes following PPA infusion. Assuming that nose poking represents exploratory behaviour, one possible explanation is that PPA infusions resulted in an increase in exploratory behaviour secondary to cognitive deficits in learning and memory. Intersession habituation, measured as a decrease in exploratory behaviour upon re-exposure to a novel environment, is considered to be an indicator of learning and memory (Leussis & Bolivar, 2006). Since PPA infusions resulted in an increase in exploratory behaviour across testing sessions, this may indicate that PPA animals failed to retain information about the novel environment. However, it is important to mention

that control animals maintained a relatively stable level of nose poking across infusion days, which would signify little or no habituation to the hole-board apparatus.

Another possible explanation for the results in the current study is that nose poking may encompass both exploratory and repetitive behaviour. Makanjuola and colleagues (1977) described exploratory behaviour as the overall pattern of nose pokes, whereas repeated responses into one hole was considered a sign of stereotypy. The hole category data from the current study allows for the assessment of the pattern of nose pokes within the apparatus, and therefore exploratory behaviour. Overall, animals made a similar proportion of corner, wall, and centre nose pokes. This suggests that PPA infusions did not alter exploratory behaviour. Although the current study did not measure repeated responses into one hole, the increase in nose poking seen in PPA animals could plausibly result from repetitive patterns of nose poking. Studies using a hole-board with 16 empty holes (similar to the current study), have shown that there is a gradual transition from exploratory to stereotyped nose poking observed across time, with most exploratory nose poking occurring within the first 10 minutes in the hole-board apparatus (Makanjuola et al., 1977; Makanjuola, Hill, Dow, Campbell, & Ashcroft, 1977). This trend was seen in both control and drug-treated animals, with control animals displaying some stereotyped nose poking (as defined by repeated responses to one hole).

Results from the current study are consistent with this interpretation. PBS animals displayed a relatively stable level of nose poking across infusion days. Across time, PBS animals showed a marked decrease in nose poking after the first 10 minutes within the hole-board apparatus. This suggests that in control animals, nose poking

represented mostly an exploratory response, as opposed to a repetitive response in the hole-board. In contrast, PPA infused animals showed an increase in nose poking behaviour across infusion days, which corresponded with a relatively consistent level of nose poking after the first 10 minutes within the apparatus, suggestive of a repetitive response.

There are a number of ways in which PPA might affect brain function to cause the behavioural impairments reported here. Histological examination of brain tissue in rodents has shown that PPA can induce an innate neuroinflammatory response, characterized by reactive astrogliosis and activated microglia in the hippocampus and neocortical white matter (MacFabe et al., 2007). Neuroinflammation is also known to occur in other diseases, such as Parkinson's and Alzheimer's disease, suggesting that this response may impair normal cognitive processes (Ferretti & Cuello, 2011; Whitton, 2007). Activated microglia secrete cytokines and toxic substances (e.g., nitric oxide) that are potentially damaging to neurons, which may impair brain function (Barron, 1995). However, fast-acting mechanisms caused by PPA, such as closure of gap junctions and pH-dependent increases in serotonin, may also explain the behavioural changes seen in the current study. Connexin-36 knock-out mice show deficits in normal spatial coding and short-term spatial memory, suggesting that electrical coupling of interneurons in the hippocampus via gap junctions are important for spatial coding and cognition (Allen, Fuchs, Jaschonek, Bannerman, & Monyer, 2011). Moreover, studies have shown that 5-HT(1A) receptor agonists produce learning and memory deficits in rodents (Ogren et al., 2008). It is possible that a fast-acting mechanism combined with neuroinflammation led to the behavioural impairments seen here.

The synthesis and release of dopamine and serotonin following intracellular acidification by PPA may explain the stereotyped nose poking behaviour seen in these animals. Increases in both serotonin and dopamine in corticostriatal circuits of the brain have been linked to stereotyped and repetitive behaviour in rodents (Langen, Kas, Staal, van Engeland, & Durston, 2011) and humans (Langen, Durston, Kas, van Engeland, & Staal, 2011). Indeed, studies using the hole-board apparatus suggest that some aspects of the dopaminergic system are involved in nose poking behaviour (Kliethermes & Crabbe, 2006).

The findings from the current study indicate that PPA induces locomotor behaviour and stereotyped/repetitive behaviour similar to the symptoms seen is ASD. Individuals with ASD often display hyperactivity, motor stereotypies, and repetitive behaviour (Matson, Dempsey, & Fodstad, 2009; Murray, 2010). Additionally, those with ASD typically display decreased exploratory behaviour compared to normal individuals when exposed to novel environments (Pierce & Courchesne, 2001). This is contradictory to results seen in PPA-infused rodents, assuming that increased nose poking should be considered increased exploratory behaviour, but this interpretation of the hole-board task is still under considerable debate.

Importantly, the proposed mechanisms underlying the behavioural impairments seen is PPA-infused animals are also theoretically linked to ASD. Aberrations in dopamine and serotonin have been reported in autistic individuals (Chugani, 2004; Previc, 2007), and treatment of repetitive behaviours has been shown to be effective with serotonin reuptake inhibitors, and serotonin and dopamine antagonists (McDougle,

Kresch, & Posey, 2000; McPheeters et al., 2011). An active neuroinflammatory process has also been observed in those with ASD (Morgan et al., 2010; Vargas et al., 2005).

In conclusion, central infusions of PPA in adult male rats resulted in locomotor abnormalities and increased repetitive behaviour compared to controls. The direct or indirect physiological properties of PPA, such as intracellular acidosis and neuroinflammatory changes, may be a plausible mechanism underlying these behavioural effects. The current findings are consistent with the symptoms seen in individuals with ASD, and taken together with previous findings using this model, support the use of PPA in an animal model of autism. Further research at critical developmental periods is needed to better understand the mechanisms responsible for the behaviours produced by PPA treatment and their potential involvement in human ASD.

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Chapter 3

Intracerebroventricular propionic acid induces perseverative behaviour in rats tested in an automated hole-board apparatus: Support for an animal model of autism spectrum disorders

3.1 Introduction

The prevalence of autism spectrum disorders (ASD), a class of neurodevelopmental disorders, has increased dramatically over the last several decades, and currently affects approximately 1 in 110 children (Boyle et al., 2011). ASD is characterized by three major behavioural symptom domains: communication deficits, abnormal social interaction, and repetitive/restricted behaviours (American Psychiatry Association, 1994). Other associated symptoms may include hyperactivity, sensitivity to sensory stimuli, cognitive deficits, seizures, and gastrointestinal (GI) disturbances (Kootz, Marinelli, & Cohen, 1982; Markram, Rinaldi, & Markram, 2007; Murray, 2010; Tuchman, Moshe, & Rapin, 2009; Williams et al., 2011). ASD has traditionally been viewed as a strongly genetic based disorder, however increasing evidence suggests that environmental, dietary, and gastrointestinal factors may also be contributing factors (Ratajczak, 2011; Williams et al., 2011).

Propionic acid (PPA) is a short-chain fatty acid (SCFA) endogenously produced as an intermediary of fatty acid metabolism and as a metabolite of bacterial fermentation in the gut (Al-Lahham, Peppelenbosch, Roelofsen, Vonk, & Venema, 2010; Thompson et al., 1990). In the GI tract, PPA is capable of altering gut motility, dilating colonic arteries, and activating mast cells (Karaki et al., 2006; Mitsui, Ono, Karaki, & Kuwahara, 2005). PPA is able to cross the gut-blood barrier and enter the systemic circulation, where it can gain access to the central nervous system (CNS) by crossing the blood-brain barrier by passive or active means (Bergersen, Rafiki, & Ottersen, 2002). Once in the CNS, PPA has widespread effects including changes in neurotransmitter synthesis and release, lipid metabolism, mitochondrial function, immune activation, and

gene expression (Brass & Beyerinck, 1988; Parab, Nankova, & La Gamma, 2007; MacFabe et al., 2008; Wyse et al., 1998). Furthermore, intracellular acidification, due to PPA accumulation within cells, can alter neurotransmitter release, inhibit gap junctions, and promote intracellular calcium release (Remblier, Pontcharraud, Tallineau, Piriou, & Huguet, 1999; Rorig, Klausa, & Sutor, 1996). All of the CNS effects of PPA have the potential to affect neuronal communication and therefore behaviour.

PPA may be associated with the etiology and pathogenesis of ASD. Anecdotal evidence from parents suggests a worsening of behavioural symptoms following consumption of refined wheat and dairy products (Jyonouchi, 2009). A recent randomized controlled trial of casein- and gluten-free diets in children with ASD supports these anecdotal reports, with an improvement in some behavioural symptoms following implementation of this diet (Whiteley et al., 2010). Interestingly, PPA is present in dairy as a result of the manufacturing process and is used as a preservative in refined wheat products, though PPA produced by enteric bacteria may also increase following consumption of these products (Brock & Buckel, 2004; Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). A subset of children with ASD, particularly those with associated GI symptoms or behavioural regression, have increased levels of the enteric bacterial species *Clostridium* and *Desulfovibrio*, both of which are known to produce PPA and other SCFAs (Finegold et al., 2002; Finegold, 2011; Parracho, Bingham, Gibson, & McCartney, 2005). Valproic acid can elevate levels of SCFAs, including PPA, and exposure to valproic acid early in development increases the risk of ASD (Ornoy, 2009). Although there are currently no studies directly measuring PPA in ASD patients, there is indirect evidence linking PPA to autism, including metabolic

impairment of fatty acids and deficiencies in carnitine and glutathione, consistent with PPA's physiological effects (Bell et al., 2004; Filipek, Juranek, Nguyen, Cummings, & Gargus, 2004; James et al., 2009).

The physiological effects of PPA and the evidence linking PPA to autism led our laboratory to propose that alterations in PPA may be related to some of the symptoms seen in ASD, and therefore we recently investigated the effects of PPA treatment in rodents as a potential animal model of ASD. We found that intracerebroventricular (ICV) infusions of PPA in adult rats resulted in hyperactivity, repetitive behaviours, kindled seizures, social impairments, cognitive deficits, altered brain phospholipid profiles, widespread oxidative stress, and an innate neuroinflammatory response (MacFabe et al., 2007, 2008; Nie et al., 2011; Shultz et al., 2009, 2008; Thomas et al., 2010). The behavioural, biochemical, electrophysiological, and neuropathological effects following PPA treatment is consistent with human findings in ASD (Bauman & Kemper, 2005; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005; Wiest, German, Harvey, Watkins, & Hertz-Picciotto, 2009). In order to assess the effects of PPA during development, our most recent work investigated the central and systemic effects of PPA in adolescent and neonatal rodents, and thus far our results have been consistent with symptoms seen in autistic patients and lend further credence to the PPA rodent model of ASD (for review of these results see Foley, Tichenoff, Ossenkopp, & MacFabe, 2010; MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011; Shams, Kavaliers, Foley, Ossenkopp, & MacFabe, 2009).

The goal of the current study was to further validate this animal model and extend the findings reported by Meeking and colleagues (2009), which assessed the

effect of central administration of two doses of PPA on locomotor and repetitive behaviour in the hole-board task. In that study, PPA treatment resulted in a dose-dependent increase in locomotor behaviour and nose poking behaviour (a measure of repetitive behaviour from the hole-board apparatus), analogous to the symptoms of ASD (Meeking, Foley, Tichenoff, Ossenkopp, & MacFabe, 2009). The current study also assessed the effects of two doses of PPA using the hole-board apparatus, but unlike the previous study, relevant olfactory stimuli (i.e. clean and soiled bedding, see Method) was placed within the wells of the hole-board apparatus as a means of measuring perseverative behaviour. It was expected that PPA treatment would result in perseveration, similar to previous findings associated with PPA and consistent with symptoms of ASD.

3.2 Method

3.2.1 Subjects

Thirty-two naive male Long-Evans rats were used, weighing 200-225 g (approximately 47-49 days old) at the time of arrival to the facility. Animals were housed individually in standard rat polypropylene cages (W 26 x L 48 x H 21 cm) with *ad libitum* access to food (LabDiet RMH 3000) and tap water in a temperature-controlled colony room (21 ± °C) on a 12:12 h light-dark cycle (lights on at 07:00 h). Behavioural testing occurred during the light phase of the cycle. Animals were left undisturbed for one week prior to the cannulation surgery. All procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the University of Western Ontario Animal Use Subcommittee.

3.2.2 Apparatus

Locomotor activity was monitored using three Versamax Animal Activity Monitors (AccuScan Model DCM-8, Columbus, OH, USA). Each monitor consisted of a clear Plexiglas open field chamber (W 40 cm x L 40 cm x H 30.5 cm) and a clear plastic lid with air holes. Movement was recorded via a grid of infrared beams located on all four sides of the chamber for horizontal activity (16 equally spaced beams 2.54 cm apart and 4.5 cm from the floor) and a grid of infrared beams located on two sides of the chamber for vertical activity (16 beams located 15 cm above the box floor). The automated activity monitors were equipped with a hole-board on the floor of the chamber to measure nose poke responses (see Figure 3.1A-D). The hole-board is an elevated platform with 16 equally spaced holes (2.54 cm diameter) with small wells (5.08 cm diameter) underneath each hole. A wire mesh screen was placed over each well (see Figure 3.1D). A set of infrared beam sensors, separate from those recording locomotor activity, are located between the well and the platform, allowing for nose poke counts for each hole to be recorded via beam breaks. VersaMax Analyzer software (Accuscan Model VSA-16, Columbus, OH) recorded data from each automated activity monitor and relayed it to a computer that stored the data for subsequent analysis. All sessions within the automated activity monitors were video-recorded and later reviewed to ensure accuracy of the computer generated nose poke data.

3.2.3 Procedure

3.2.3.1 Surgery. To induce anaesthesia, animals were placed into a sealed plastic box into which 4% isoflurane at 2 L/min oxygen was introduced. The animal was then placed into a Kopf stereotaxic device equipped with a gas flow mask delivering 2.5%

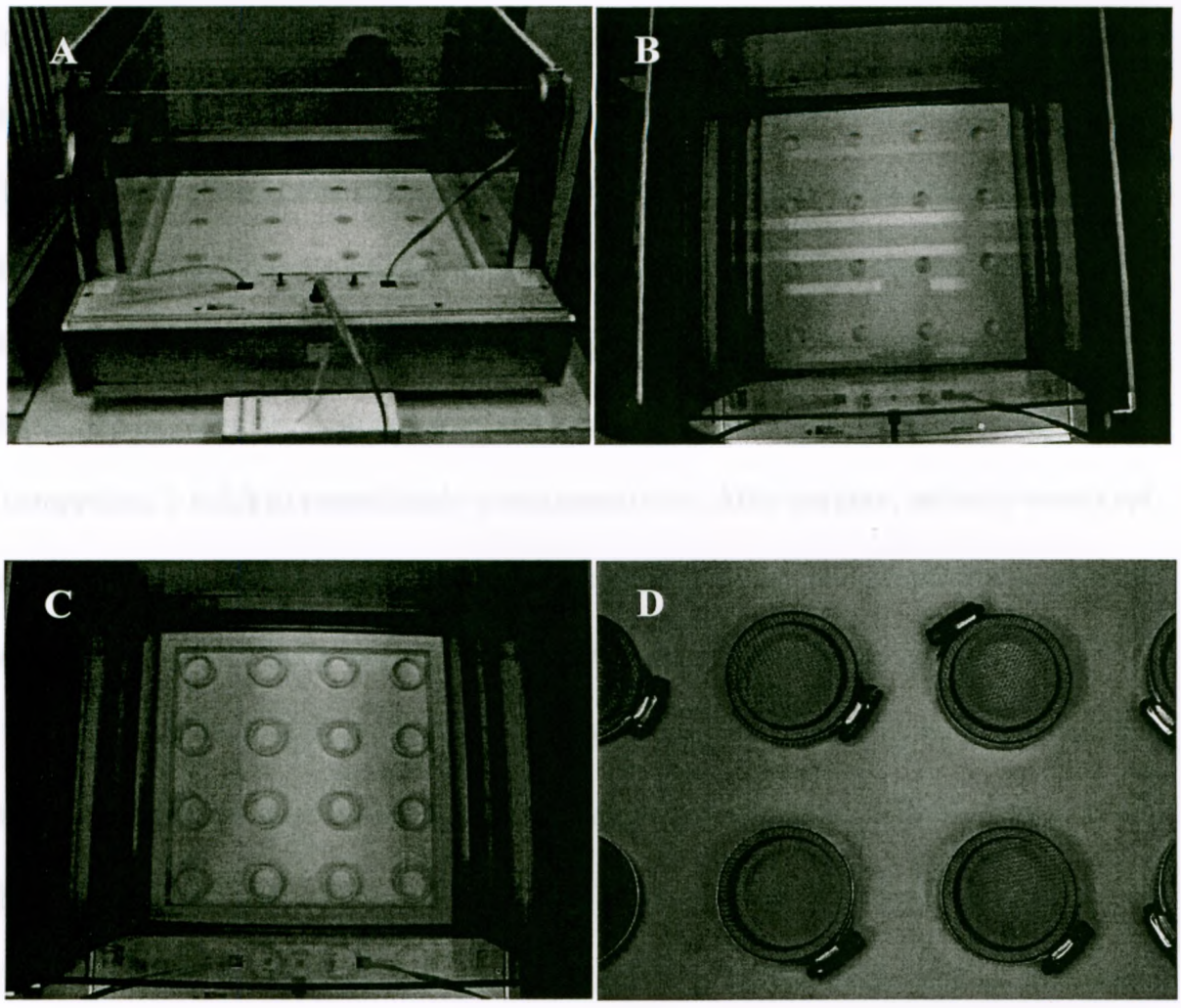

Figure 3.1. Photographs of the automated activity monitors equipped with a hole-board on the floor of the chamber. (A) Front view; (B) Top-down view; (C) Top-down view with platform removed to show the 16 equally spaced wells beneath the floor of the chamber; (D) Close-up of wells with wire mesh screens.

isoflurane at 500 mL/min of oxygen to maintain anaesthesia during surgery. Under aseptic conditions, right lateral ventricular cannulation surgery was performed (AP -1.4 mm, ML -1.8 mm, DV -3.0 mm). Each animal was implanted with a 23-gauge guide cannula with the tip in the right lateral ventricle in accordance with a standard rat atlas (Paxinos & Watson, 1998). The indwelling cannula was secured chronically using dental acrylic anchored in place with small stainless steel screws inserted into the skull. A removable obturator sealed the guide cannula and was only removed for infusions during the experiment. Animals received a subcutaneous injection of analgesic (ketoprofen, 1 mL/kg) immediately post-operatively. After surgery, animals were kept warm under a heating lamp until righting responses and locomotion returned. Animals were housed individually and allowed two weeks recovery prior to testing.

3.2.3.2 Treatment groups and infusion procedure. Following recovery, animals were randomly assigned to one of three groups: high-dose PPA (0.26 M, 4.0 μL, n = 11), low-dose PPA (0.052 M, 4.0 μL, n = 10), or phosphate buffered saline vehicle (PBS, 4.0 μL, n = 11). Propionic acid was dissolved in PBS vehicle, and all solutions were buffered to pH 7.5 using concentrated HCL or NaOH. Each animal received intracerebroventricular (ICV) infusions twice daily (separated by 4 hours) for seven consecutive days. The first infusion occurred during the light phase at 09:00 h. Solutions were infused using a 30 gauge injection cannula that was connected to a Sage syringe pump with sterile PE10 tubing. The tip of the injection cannula protruded 0.5 mm beyond the tip of the guide cannula. The syringe pump dispensed 4.0 μL of solution over a 60 s interval, and the injection cannula remained in place for an additional 60 s before being removed.

3.2.3.3 Testing. Following two weeks of recovery, rats were handled and habituated to the automated activity monitors for two days (30 minutes per day). On the third day, baseline levels of activity and nose poke responses were recorded in the absence of infusion. During the seven treatment days, animals were placed in the automated monitors following the second infusion of the day for 30 minutes to record locomotor activity and nose poke counts (six 5 minute time bins). All wells within the hole-board platform remained empty with wire mesh screens for habituation, baseline, and the first two treatments days. On treatment days 3 through 6, clean bedding was placed in one centre well and soiled bedding was placed in another centre well (holes 6 and 11, counter-balanced within treatment groups, see Figure 2). All animals were exposed to soiled bedding from one unfamiliar male rat (i.e., bedding from an individually housed rat in a separate colony room soiled for 72 hours) on each of treatment days 3 to 6. The soiled bedding was from a different unfamiliar male rat for each particular treatment day (i.e., soiled bedding was used from 4 different unfamiliar male rats, one for each of days 3 through 6). On the seventh treatment day, the soiled bedding was from the experimental rat's own cage (also soiled for 72 hours). Rats were weighed daily to monitor health.

3.2.3.4 Perfusions. The day after behavioural testing was completed, rats were euthanized via intraperitoneal injection (euthanyl 270 g/mL, ~0.5 mL per animal). Animals were transcardially perfused with a PBS/4% paraformaldehyde solution. The fixed brains were removed and kept in a sucrose solution for localization of the indwelling cannula.

3.2.4 Behavioural Measures

Locomotor activity was analyzed using eight distinct measures. The horizontal activity measures analyzed were: total distance – total horizontal distance (cm) travelled; horizontal movement time – amount of time (s) an animal was engaged in horizontal movement; and number of horizontal movements – the number of horizontal movements separated by a 1 s stop time. The vertical activity measures analyzed were: vertical movement time – amount of time (s) an animal spent in a vertical position; and number of vertical movements – number of vertical movements separated by a 1 s stop time. The repetitive locomotor measures were: clockwise revolutions – the numbers of times an animal moved around in a clockwise circle of at least 2 inches in diameter; counterclockwise revolutions – the number of times an animal moved around in a counterclockwise circle of at least 2 inches in diameter; and the number of stereotypic movements – repeated breaking of the same infrared beam separated by 1 sec or more.

Nose poke behaviour was analyzed using total nose poke counts, hole category, and difference scores. Total nose poke counts are the total number of nose pokes across an entire testing session (includes all 16 different holes in the hole-board). Hole category data classified the holes into four categories based on location within the open-field apparatus in order to determine if there was a pattern of hole preference. The four hole categories based on location included corner holes (1, 4, 13, and 16); centre holes (6, 7, 10, and 11), and wall holes (2, 3, 5, 8 9, 12, 14, and 15) (see Figure 3.2). In order to determine preferences between the soiled and clean bedding across treatment days, difference scores were calculated. The total number of nose pokes at the hole with soiled bedding (SB) was calculated as a percent of total nose pokes for that particular testing

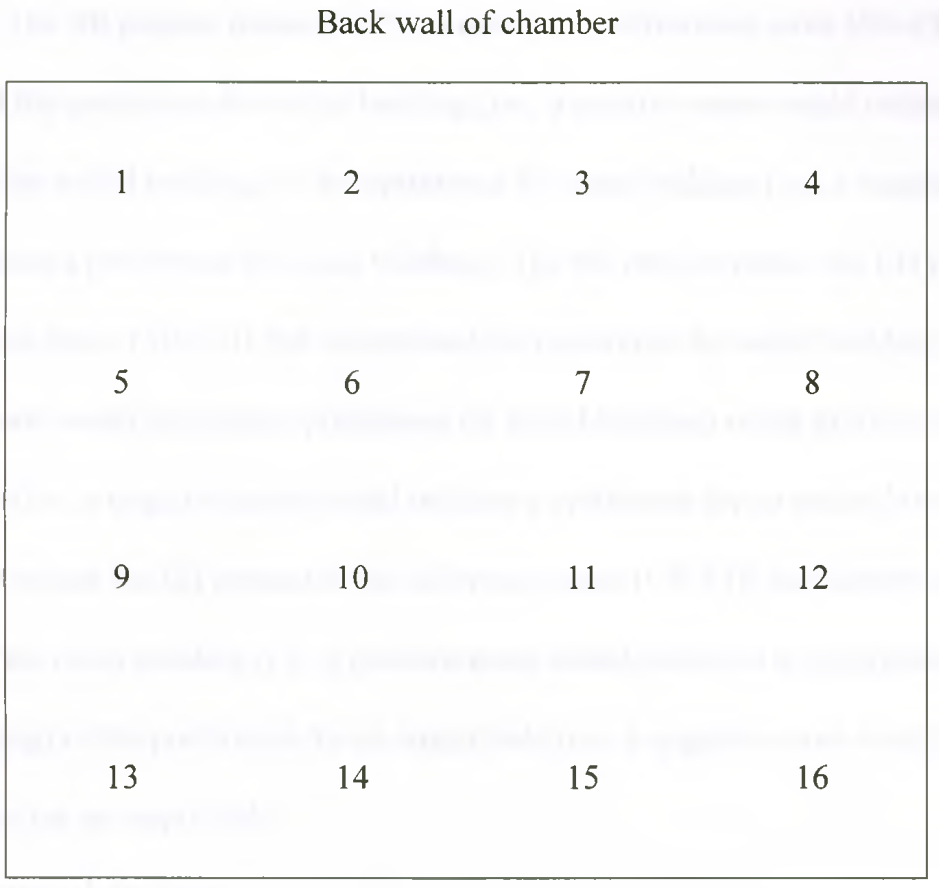

Figure 3.2. Schematic of the hole-board numbering system in the automated activity monitor, indicating four corner holes (1, 4, 13, and 16), four centre holes (6, 7, 10, and 11), and eight wall holes (2, 3, 5, 8, 9, 12, 14, and 15).

session for each individual animal to control for differences in total nose pokes between animals. The same was done for the hole with clean bedding (CB) and a nearby empty hole (EH). The SB percent minus the CB percent is the difference score (SB-CB) that determined the preference for soiled bedding (i.e., a positive score would indicated a preference for soiled bedding) or the preference for clean bedding (i.e., a negative score would indicate a preference for clean bedding). The SB percent minus the EH percent is the difference score (SB-EH) that determined the preference for soiled bedding (i.e., a positive score would indicated a preference for soiled bedding) or the preference for an empty hole (i.e., a negative score would indicate a preference for an empty hole). The CB percent minus the EH percent is the difference score (CB-EH) that determined the preference for clean bedding (i.e., a positive score would indicated a preference for clean bedding) or the preference for an empty hole (i.e., a negative score would indicate a preference for an empty hole).

3.2.5 Statistical Analysis

Data were analyzed for main effects and interactions using a repeated measures split-plot analysis of variance (ANOVA) with drug treatment (PBS, low-dose PPA, and high-dose-PPA) as the between-subjects factor and infusion day (7 infusions days) and time block (six 5 minute time blocks) as the within subjects factors (with the exception of nose poke counts, hole category, and difference scores, which were analyzed with only infusion day as the within subjects factor). Hole category and difference score data were analyzed using separate ANOVAs for each set of infusion days (i.e., days 1 to 2, days 3 to 6, and day 7). The dependent variables were several locomotor and nose poke variables. Separate statistical analyses were conducted for each variable. A one-way

ANOVA was conducted on the baseline data to ensure that there were no individual differences prior to treatment days. For any variable in which the baseline ANOVA indicated significant group differences, a repeated measures ANCOVA was performed, using the baseline data as a co-variate. Where appropriate, post-hoc comparisons were conducted using Tukey`s HSD or Sidak. Significance was set to $\alpha = .05$.

3.3 Results

3.3.1 Cannula Placement

Histological analysis showed that cannula tips were correctly located within the right lateral ventricle in all animals (see Figure 3.3).

3.3.2 Locomotor Variables

3.3.2.1 Baseline. A one-way ANOVA was performed on the baseline data for each locomotor variable. The one-way ANOVAs were not significant for total distance travelled, horizontal movement time, the vertical activity measures (number of vertical movements and vertical movement time), or any of the repetitive activity measures (number of stereotypic movements, number of clockwise revolutions, and number of counterclockwise revolutions). Thus, there were no differences in locomotor activity among the treatment groups for these behavioural measures prior to the first infusion day. The one-way ANOVA was significant on baseline day for number of horizontal movements; therefore, baseline data were used as a covariate on infusion days for this variable.

3.3.2.2 Horizontal activity measures. The ANCOVA revealed a significant day x treatment interaction for number of horizontal movements, $F(12, 168) = 6.48$, $p < .001$. ANOVAs showed a significant main effect of treatment for horizontal movement time,

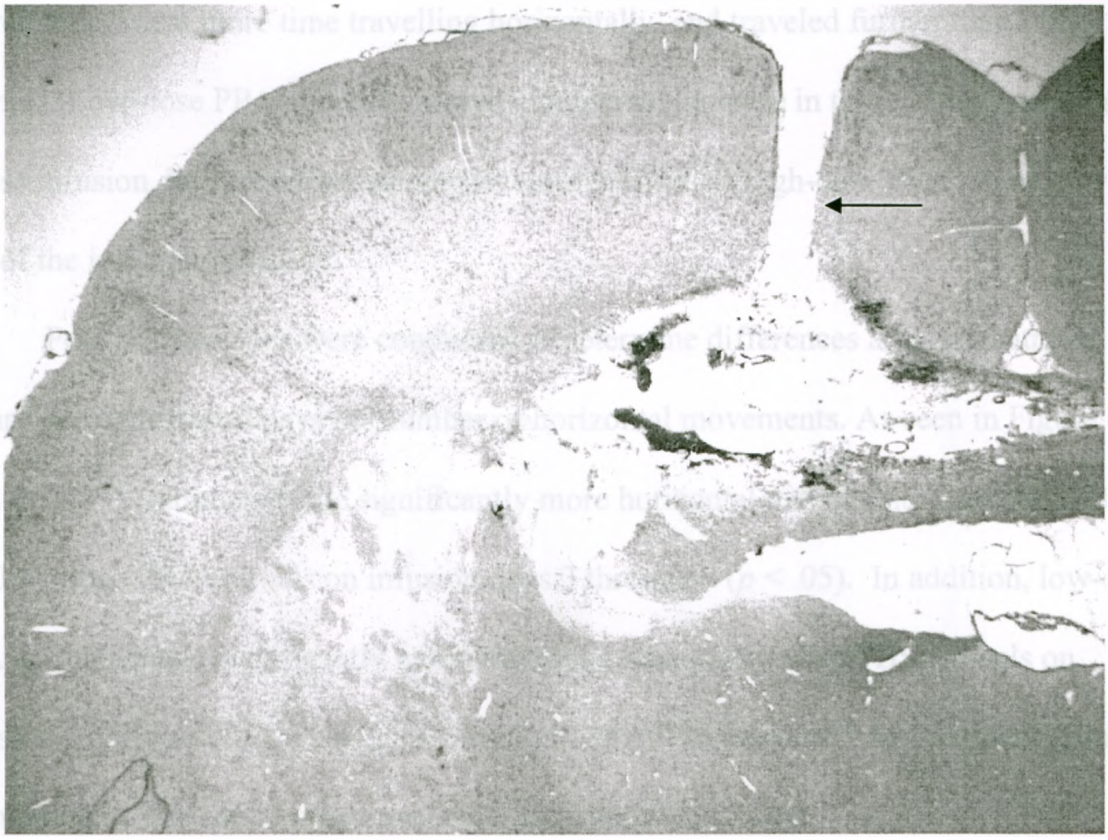

Figure 3.3. Representative photomicrograph showing proper cannula placement (arrow) into the right lateral ventricle using an H&E stain

$F(2, 29) = 21.64$, $p < .001$, and for total distance traveled, $F(2, 29) = 19.78$, $p < .001$. In general PPA infusions resulted in a dose dependent increase in horizontal activity measures. Across infusion days, high-dose PPA animals made more horizontal movements, spent more time travelling horizontally, and traveled further than PBS animals. Low-dose PPA animals showed a moderate increase in these same measures across infusion days, reaching somewhat similar levels as high-dose PPA animals by the end of the infusion schedule.

Post-hoc analyses were conducted to determine differences among treatment groups across infusion days for number of horizontal movements. As seen in Figure 3.4, high-dose PPA animals made significantly more horizontal movements than both PBS and low-dose PPA animals on infusion days 3 through 7 ($p < .05$). In addition, low-dose PPA animals made significantly more horizontal movements than PBS animals on infusion day 6 ($p < .05$). There were no significant differences among treatment groups for number of horizontal movements on infusions days 1 and 2.

Across the 30 minute testing sessions, there was a significant time x treatment interaction for number of horizontal movements, $F(10, 140) = 14.15$, $p < .001$. Post-hoc analyses were conducted across the six 5 minute time bins for each infusion day. On infusion day 1, there were no significant differences among groups for number of horizontal movements for the entire 30 minute testing session (data not shown). On infusion day 4, high-dose PPA animals made significantly more horizontal movements than both PBS animals during 3 time bins (time 10-15 and time 20-30, $p < .05$) and also low-dose PPA animals during 2 time bins (time 20-30, $p < .05$; data not shown). There were no significant differences among treatment groups for number of horizontal

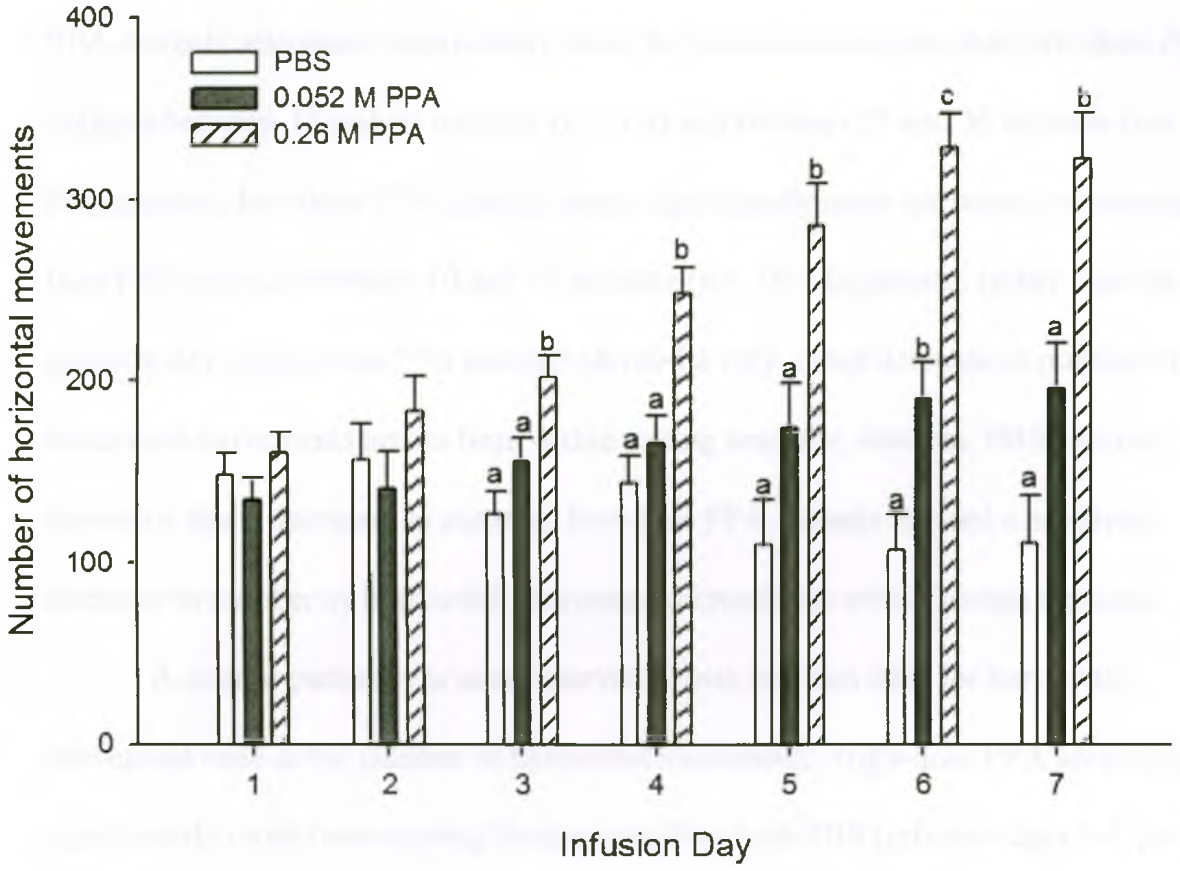

Figure 3.4. Mean number of horizontal movements + *SEM* across infusion days in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a, b, or c) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

movements during the remainder of the testing session. On infusion day 7, high-dose PPA animals made significantly more horizontal movements than PBS animals during the last 25 minutes of the testing session (time 5-30, $p < .05$; data not shown). High-dose PPA animals also made significantly more horizontal movements than low-dose PPA animals between 15 and 20 minutes ($p < .05$) and between 25 and 30 minutes ($p < .05$). Furthermore, low-dose PPA animals made significantly more horizontal movements than PBS animals between 10 and 15 minutes ($p < .05$). In general, (other than the first infusion day), high-dose PPA animals showed a very minor decrease in number of horizontal movements across time within testing sessions; whereas, PBS animals showed a sharp decrease. In contrast, low-dose PPA animals showed a moderate decrease in number of horizontal movements across time within testing sessions.

A similar pattern was also observed across infusion days for horizontal movement time as for number of horizontal movements. High-dose PPA animals spent significantly more time moving horizontally than both PBS (infusion days 3-7, $p < .05$) and low-dose PPA animals (infusion days 4-6, $p < .05$; see Figure 3.5A). Low-dose PPA animals also spent significantly more time moving horizontally than PBS animals on infusion day 7 ($p < .05$). There were no significant differences among treatment groups for horizontal movement time for the first two infusion days.

Across the 30 minute testing sessions, there was a significant time x treatment interaction for horizontal movement time, $F(10, 145) = 3.58$, $p < .01$. Post-hoc analyses conducted across the six 5 minute time bins revealed that on infusion day 1, there were no significant differences among treatment groups for horizontal movement time across the entire testing session (see Figure 3.5B). On infusion day 4, high-dose PPA animals

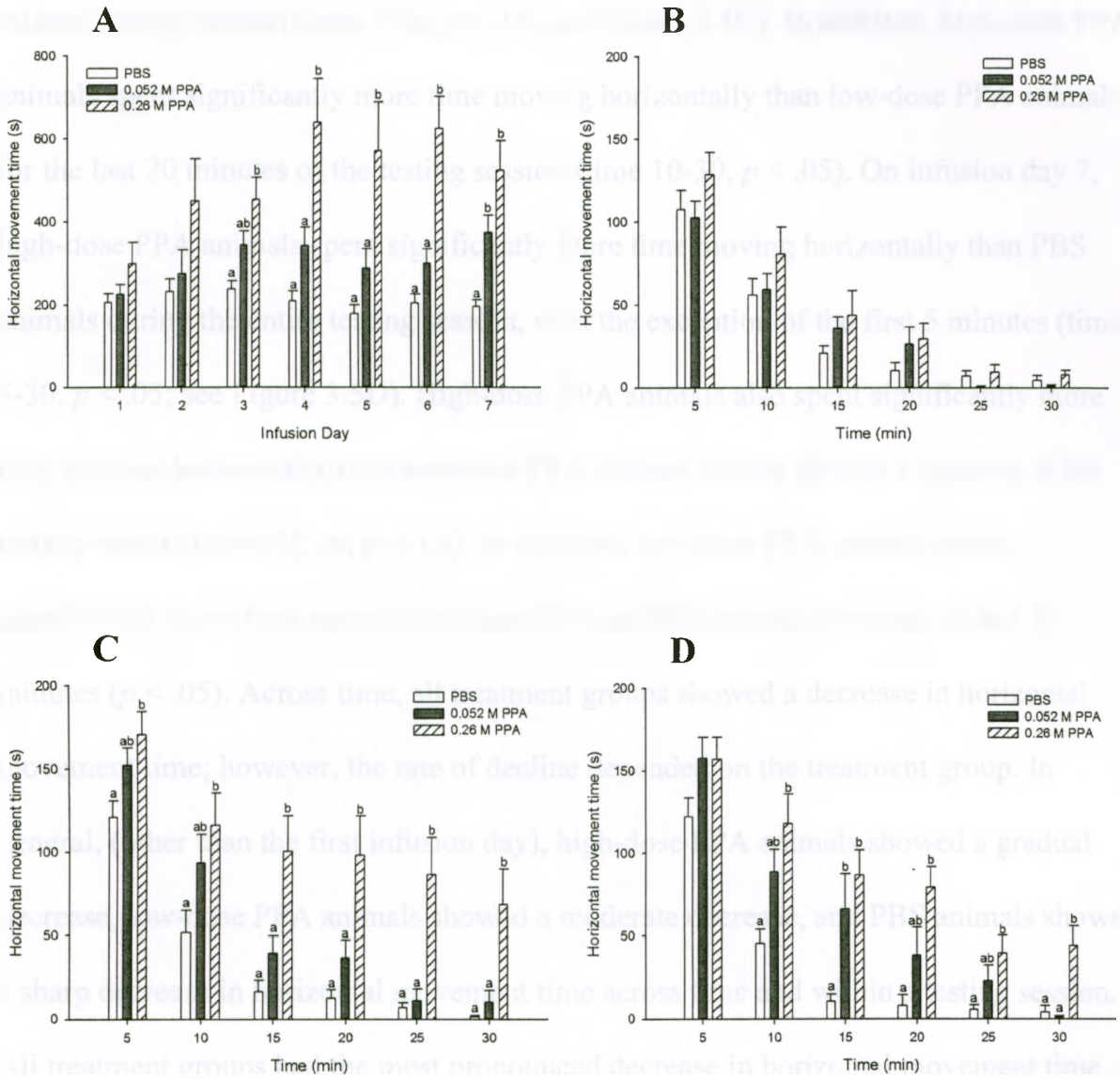

Figure 3.5. Mean horizontal movement time (s) + *SEM* across infusion days (A) or across time (B. infusion day 1; C. infusion day 4; D. infusion day 7) in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day or that particular time ($p < .05$; no superscripts indicate no significant difference between groups).

spent significantly more time moving horizontally than PBS animals during the entire 30 minute testing session (time 0-30, $p < .05$; see Figure 3.5C). In addition, high-dose PPA animals spent significantly more time moving horizontally than low-dose PPA animals for the last 20 minutes of the testing session (time 10-30, $p < .05$). On infusion day 7, high-dose PPA animals spent significantly more time moving horizontally than PBS animals during the entire testing session, with the exception of the first 5 minutes (time 5-30, $p < .05$; see Figure 3.5D). High-dose PPA animals also spent significantly more time moving horizontally than low-dose PPA animals during the last 5 minutes of the testing session (time 25-30, $p < .05$). In addition, low-dose PPA animals spent significantly more time moving horizontally than PBS animals between 10 and 15 minutes ($p < .05$). Across time, all treatment groups showed a decrease in horizontal movement time; however, the rate of decline depended on the treatment group. In general, (other than the first infusion day), high-dose PPA animals showed a gradual decrease, low-dose PPA animals showed a moderate decrease, and PBS animals showed a sharp decrease in horizontal movement time across time and within a testing session. All treatment groups had the most pronounced decrease in horizontal movement time within the first 15 minutes of a testing session, while remaining relatively stable for the last 15 minutes of a testing session.

Post-hoc analyses across infusion days for total distance travelled were similar to number of horizontal movements and horizontal movement time. On infusion days 3 through 7, high-dose PPA animals traveled significantly further than PBS animals ($p < .05$; see Figure 3.6). Furthermore, high-dose PPA animals traveled significantly further than low-dose PPA animals on infusion days 4 and 6 ($p < .05$). Low-dose PPA animals

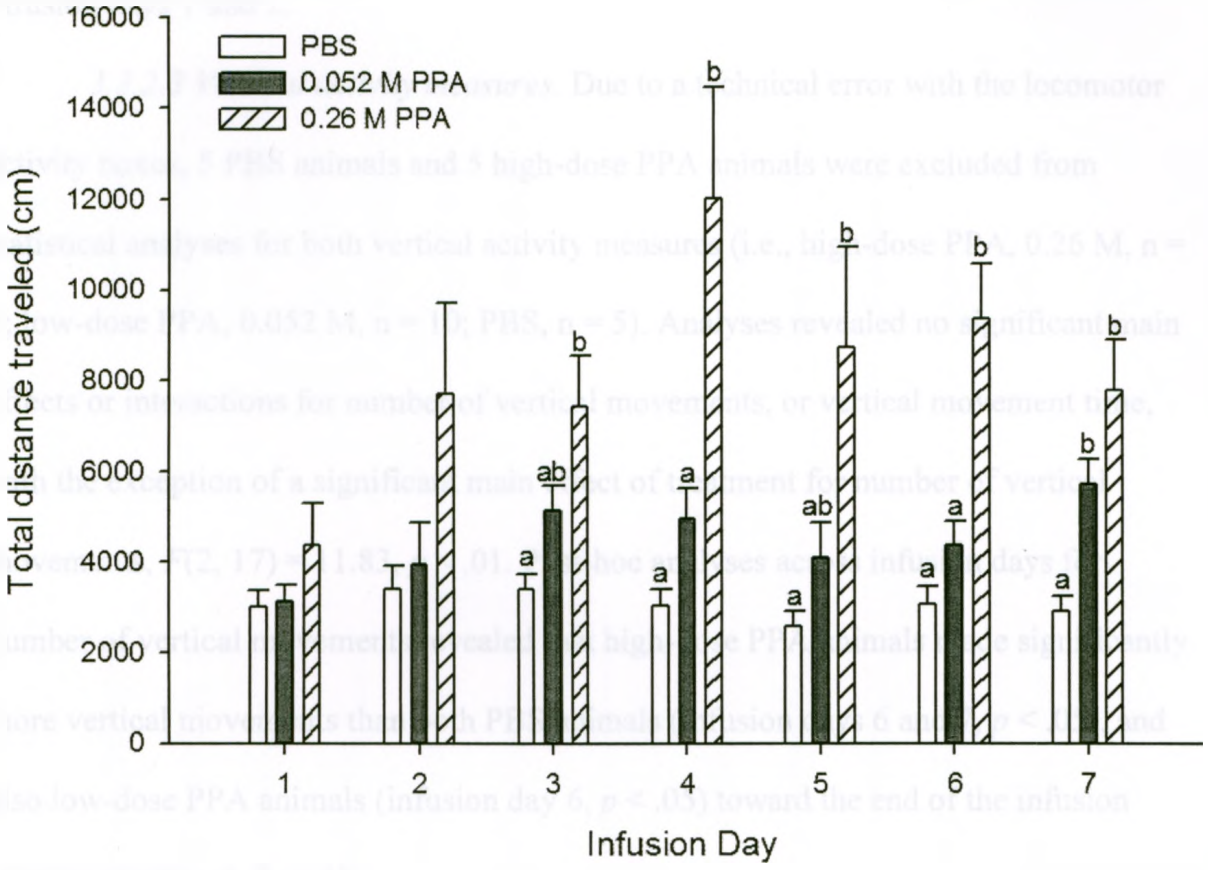

Figure 3.6. Mean total distance traveled (cm) + *SEM* across infusion days in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

traveled significantly further than PBS animals on the last infusion day ($p < .05$). There were no significant differences among treatment groups for total distance traveled on infusion days 1 and 2.

3.3.2.3 Vertical activity measures. Due to a technical error with the locomotor activity boxes, 5 PBS animals and 5 high-dose PPA animals were excluded from statistical analyses for both vertical activity measures (i.e., high-dose PPA, 0.26 M, n = 5; low-dose PPA, 0.052 M, n = 10; PBS, n = 5). Analyses revealed no significant main effects or interactions for number of vertical movements, or vertical movement time, with the exception of a significant main effect of treatment for number of vertical movements, $F(2, 17) = 11.83$, $p < .01$. Post-hoc analyses across infusion days for number of vertical movements revealed that high-dose PPA animals made significantly more vertical movements than both PBS animals (infusion days 6 and 7, $p < .05$), and also low-dose PPA animals (infusion day 6, $p < .05$) toward the end of the infusion schedule (data not shown).

3.3.2.4 Repetitive activity measures. Analyses showed a significant main effect of treatment for number of stereotypic movements, $F(2, 29) = 19.26$, $p < .001$. As seen in Figure 3.7A, PPA infusions resulted in an increase in stereotypic movements for high-dose animals, but not for low-dose animals. Post-hoc analyses across infusion days showed that high-dose PPA animals made significantly more stereotypic movements than PBS animals on infusion days 3 through 7 ($p < .05$). High-dose PPA animals also made significantly more stereotypic movements than low-dose PPA animals on infusion days 4 to 7 ($p < .05$). There were no significant differences among treatment groups for number of stereotypic movements for infusion days 1 and 2.

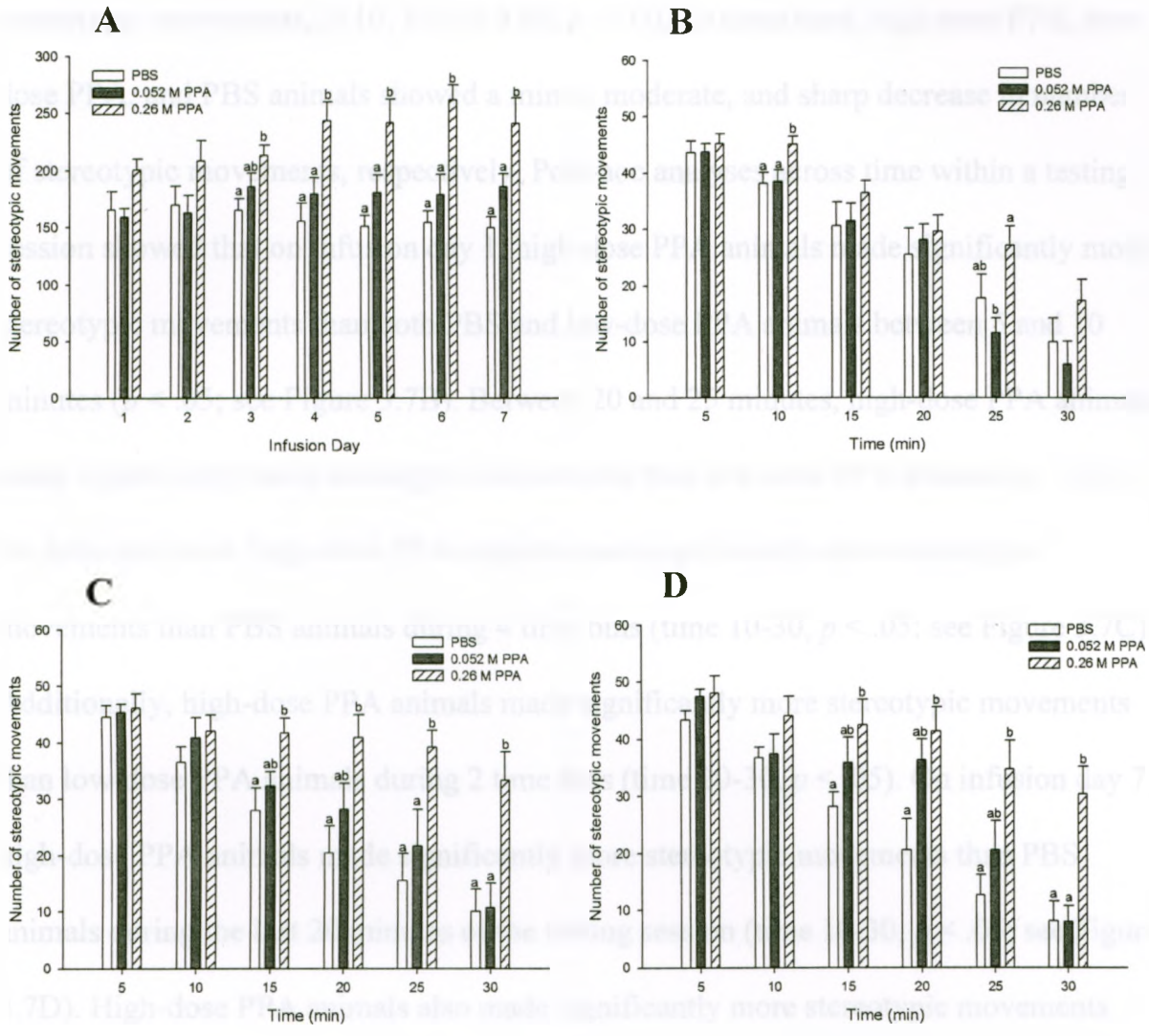

Figure 3.7. Mean number of stereotypic movements + *SEM* across infusion days (A) or across time (B. infusion day 1; C. infusion day 4; D. infusion day 7) in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day or that particular time ($p < .05$; no superscripts indicate no significant difference between groups).

There was also a significant time x treatment interaction for number of stereotypic movements, $F(10, 145) = 9.84$, $p < .001$. Across time, high-dose PPA, low-dose PPA, and PBS animals showed a minor, moderate, and sharp decrease in number of stereotypic movements, respectively. Post-hoc analyses across time within a testing session showed that on infusion day 1, high-dose PPA animals made significantly more stereotypic movements than both PBS and low-dose PPA animals between 5 and 10 minutes ($p < .05$; see Figure 3.7B). Between 20 and 25 minutes, high-dose PPA animals made significantly more stereotypic movements than low-dose PPA animals ($p < .05$). On infusion day 4, high-dose PPA animals made significantly more stereotypic movements than PBS animals during 4 time bins (time 10-30, $p < .05$; see Figure 3.7C). Additionally, high-dose PPA animals made significantly more stereotypic movements than low-dose PPA animals during 2 time bins (time 20-30, $p < .05$). On infusion day 7, high-dose PPA animals made significantly more stereotypic movements than PBS animals during the last 20 minutes of the testing session (time 10-30, $p < .05$; see Figure 3.7D). High-dose PPA animals also made significantly more stereotypic movements than low-dose PPA animals during the last 5 minutes of the testing session (time 25-30; $p < .05$).

Analyses revealed a significant main effect of treatment for clockwise, $F(2, 29) = 12.51$, $p < .001$, and counterclockwise revolutions, $F(2, 29) = 14.00$, $p < .001$. There were no significant day x treatment or time x treatment interactions for clockwise and counterclockwise revolutions. In general, high-dose PPA animals made more revolutions than PBS animals. In contrast, low-dose PPA animals were similar to PBS animals for number of revolutions. Post-hoc analyses conducted across infusion days

showed that high-dose PPA animals made significantly more clockwise revolutions than PBS animals (infusion days 3 to 7, $p < .05$; see Figure 3.8) and low-dose PPA animals (infusion days 1, and 4 to 7, $p < .05$). A somewhat similar pattern was seen across infusion days for counterclockwise revolutions (data not shown). High-dose PPA animals made significantly more counterclockwise revolutions than PBS (infusion days 3, 4, 6, and 7, $p < .05$) and low-dose PPA animals (infusion days 4 and 6, $p < .05$).

3.3.3 Nose Poke Variables

3.3.3.1 Nose poke counts. The one-way ANOVA performed on the baseline data for total nose poke counts failed to show any significant effects. Therefore, there were no significant differences among treatment groups in number of nose pokes prior to the first infusion day.

Analysis revealed a significant main effect of treatment for number of nose pokes, $F(2, 29) = 4.49$, $p < .05$. As seen in Figure 3.9, PPA infusions resulted in an increase in number of nose pokes, but post-hoc analyses across infusion days showed no significant differences among treatment groups on any infusion day except day 6 where low-dose PPA animals made significantly more nose pokes than PBS animals ($p < .05$).

3.3.3.2 Hole category. A one-way ANOVA was performed on the baseline data for each hole category variable (i.e., corner, wall, and centre nose pokes). The ANOVA was not significant for number of corner or wall nose pokes, but was significant for number of centre nose pokes. Therefore, baseline data was used as a covariate for analysis for centre nose pokes.

The ANOVAs for corner nose pokes and wall nose pokes on infusion days 1 and 2 and infusion days 3 through 6 showed no significant main effect of day or main effect

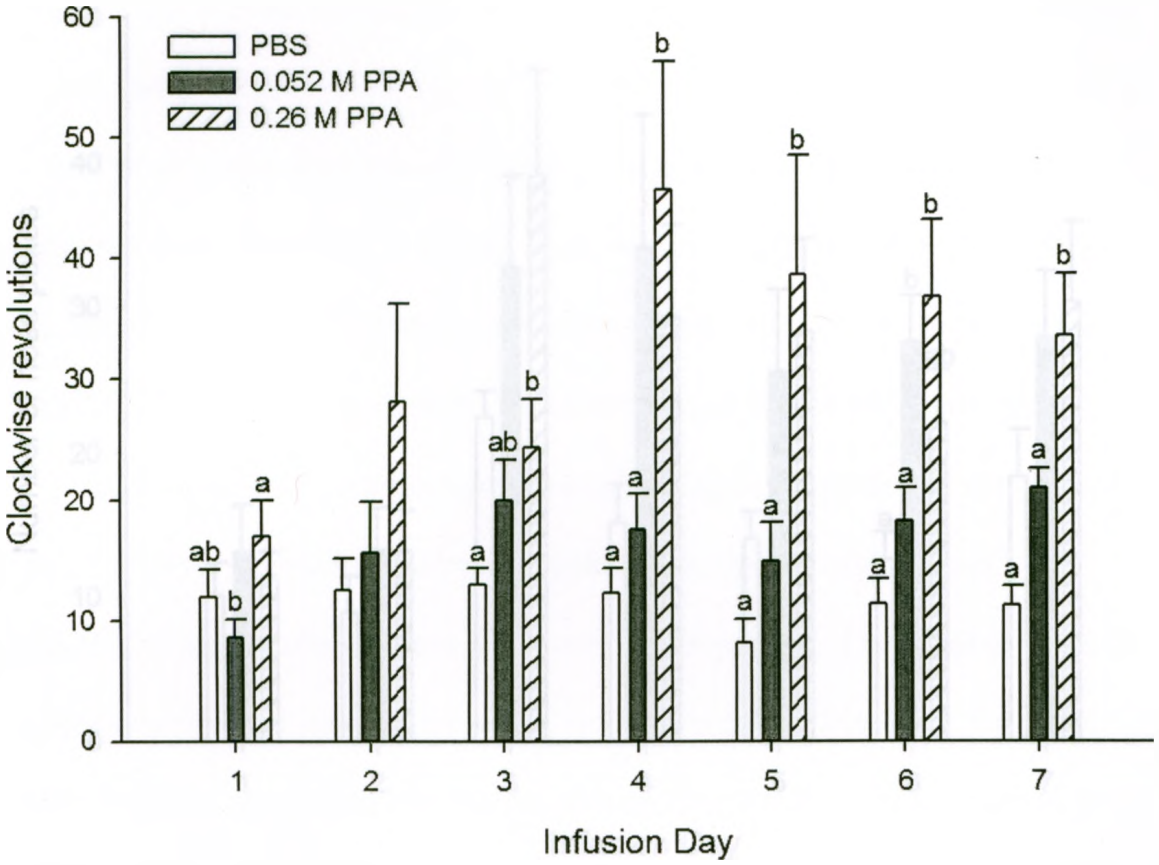

Figure 3.8. Mean number of clockwise revolutions + *SEM* across infusion days in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

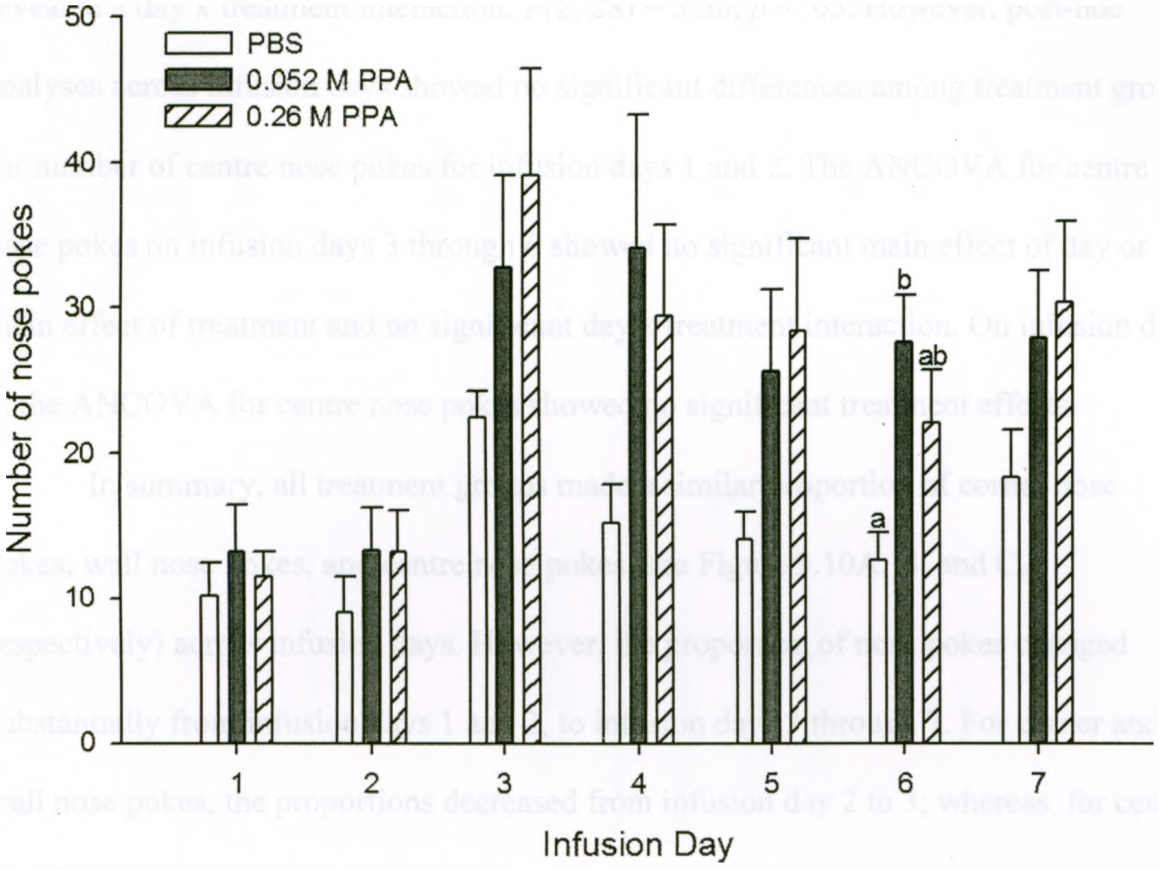

Figure 3.9. Mean number of nose pokes + *SEM* across infusion days in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26 M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatments on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups).

of treatment and no significant day x treatment interaction. On infusion day 7, the one-way ANOVAs for corner nose pokes and wall nose pokes showed no significant treatment effects. The ANCOVA for centre nose pokes on infusion days 1 and 2 revealed a day x treatment interaction, $F(2, 28) = 3.50$, $p < .05$. However, post-hoc analyses across infusion days showed no significant differences among treatment groups for number of centre nose pokes for infusion days 1 and 2. The ANCOVA for centre nose pokes on infusion days 3 through 6 showed no significant main effect of day or main effect of treatment and no significant day x treatment interaction. On infusion day 7, the ANCOVA for centre nose pokes showed no significant treatment effects.

In summary, all treatment groups made a similar proportion of corner nose pokes, wall nose pokes, and centre nose pokes (see Figure 3.10A, B, and C, respectively) across infusion days. However, the proportion of nose pokes changed substantially from infusion days 1 and 2, to infusion days 3 through 7. For corner and wall nose pokes, the proportions decreased from infusion day 2 to 3; whereas, for centre nose pokes the proportions increased.

3.3.3.3 Hole preference. The repeated measures ANOVAs for the SB-CB difference score (i.e., soiled bedding percent minus clean bedding percent; see Method section for details) on infusion days 1 and 2 and infusion days 3 through 6 showed no significant main effect of day or main effect of treatment and no significant day x treatment interaction. On infusion day 7, the one-way ANOVA for the SB-CB difference score showed that the effect of treatment was significant, $F(2, 29) = 5.56$, $p < .01$. Post-hoc analyses among treatment groups revealed that both PPA groups had

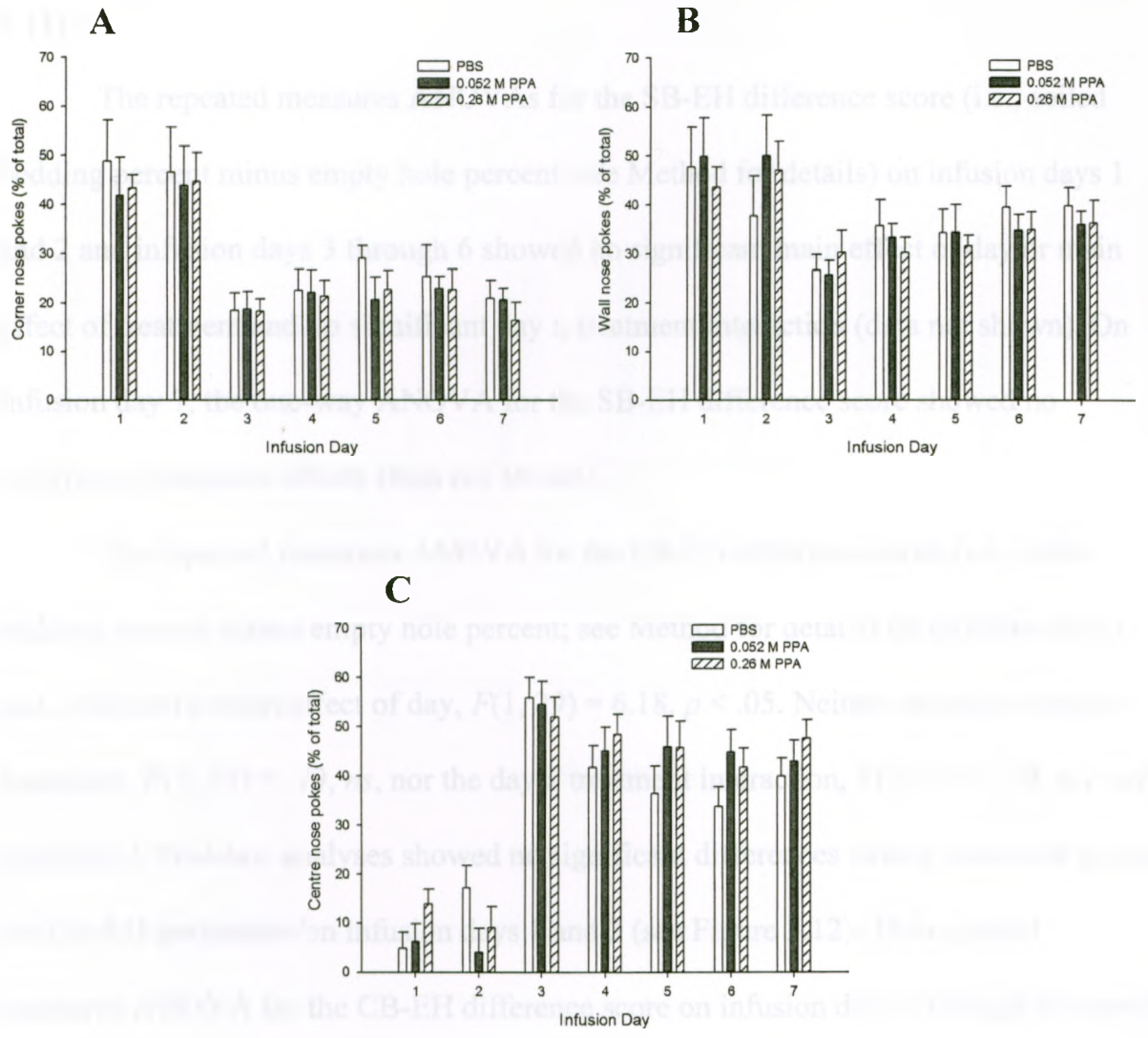

Figure 3.10. Mean number of corner nose pokes (A), wall nose pokes (B), or centre nose pokes (C) expressed as a percent of total nose pokes + *SEM* across infusion days in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups.

had significantly higher SB-CB difference scores than PBS animals ($p < .05$; see Figure 3.11).

The repeated measures ANOVAs for the SB-EH difference score (i.e., soiled bedding percent minus empty hole percent; see Method for details) on infusion days 1 and 2 and infusion days 3 through 6 showed no significant main effect of day or main effect of treatment and no significant day x treatment interaction (data not shown). On infusion day 7, the one-way ANOVA for the SB-EH difference score showed no significant treatment effects (data not shown).

The repeated measures ANOVA for the CB-EH difference score (i.e., clean bedding percent minus empty hole percent; see Method for details) on infusion days 1 and 2 showed a main effect of day, $F(1, 29) = 6.18$, $p < .05$. Neither the main effect of treatment, $F(2, 29) = .79$, *ns*, nor the day x treatment interaction, $F(2, 29) = .59$, *ns*, were significant. Post-hoc analyses showed no significant differences among treatment groups for CB-EH preference on infusion days 1 and 2 (see Figure 3.12). The repeated measures ANOVA for the CB-EH difference score on infusion days 3 through 6 showed a day x treatment interaction, $F(6, 87) = 3.05$, $p < .01$. Post-hoc analyses revealed that low-dose PPA animals had significantly greater CB-EH difference scores than high-dose PPA animals on infusion day 5 ($p < .05$; see Figure 3.12). On infusion day 7, the one-way ANOVA for the CB-EH difference score showed a significant effect of treatment, $F(2, 29) = 4.84$, $p < .05$. Post-hoc analysis showed that PBS animals had significantly higher CB-EH difference scores than both PPA groups on infusion day 7 ($p < .05$; see Figure 3.12).

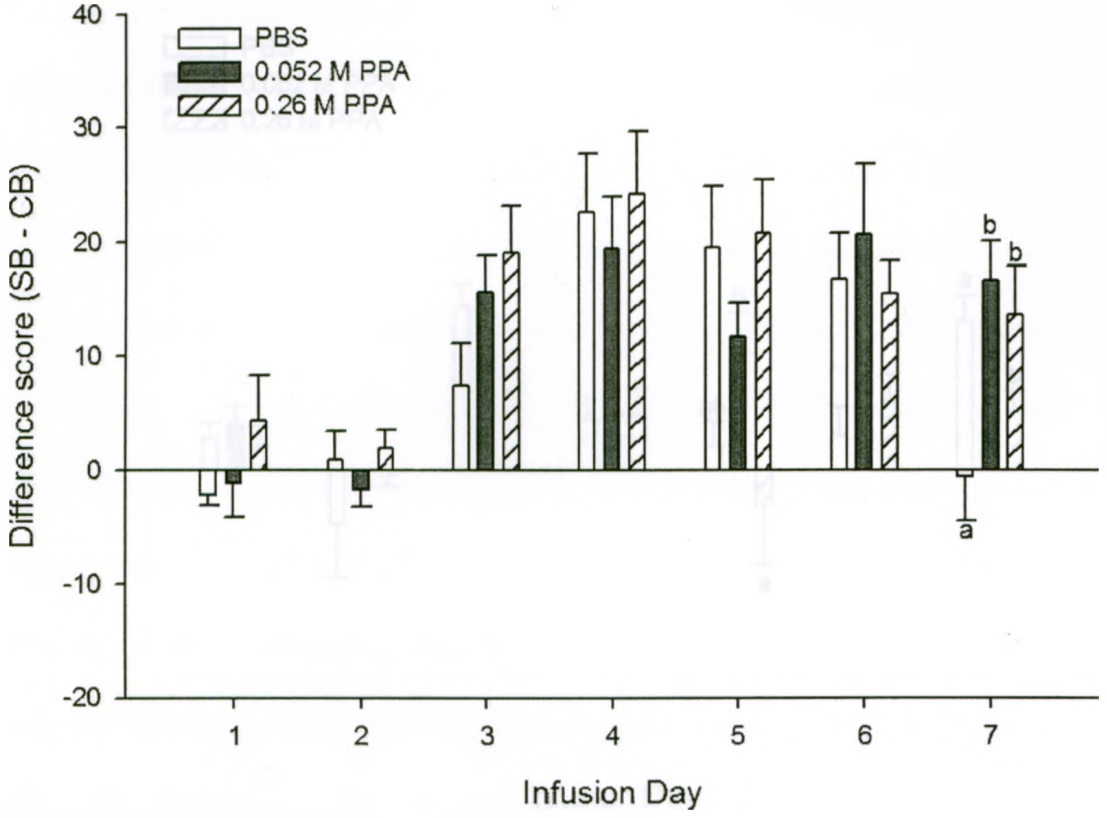

Figure 3.11. Mean difference score (SB-CB) + *SEM* across infusion days in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups). The total number of nose pokes at the hole with soiled bedding (SB) was calculated as a percent of total nose pokes for that particular infusion day for each individual animal. The same was done for the hole with clean bedding (CB). The SB percent minus the CB percent is the difference score (SB-CB) that determines the preference for soiled bedding (i.e., a positive score indicates a preference for soiled bedding) or the preference for clean bedding (i.e., a negative score indicates a preference for clean bedding).

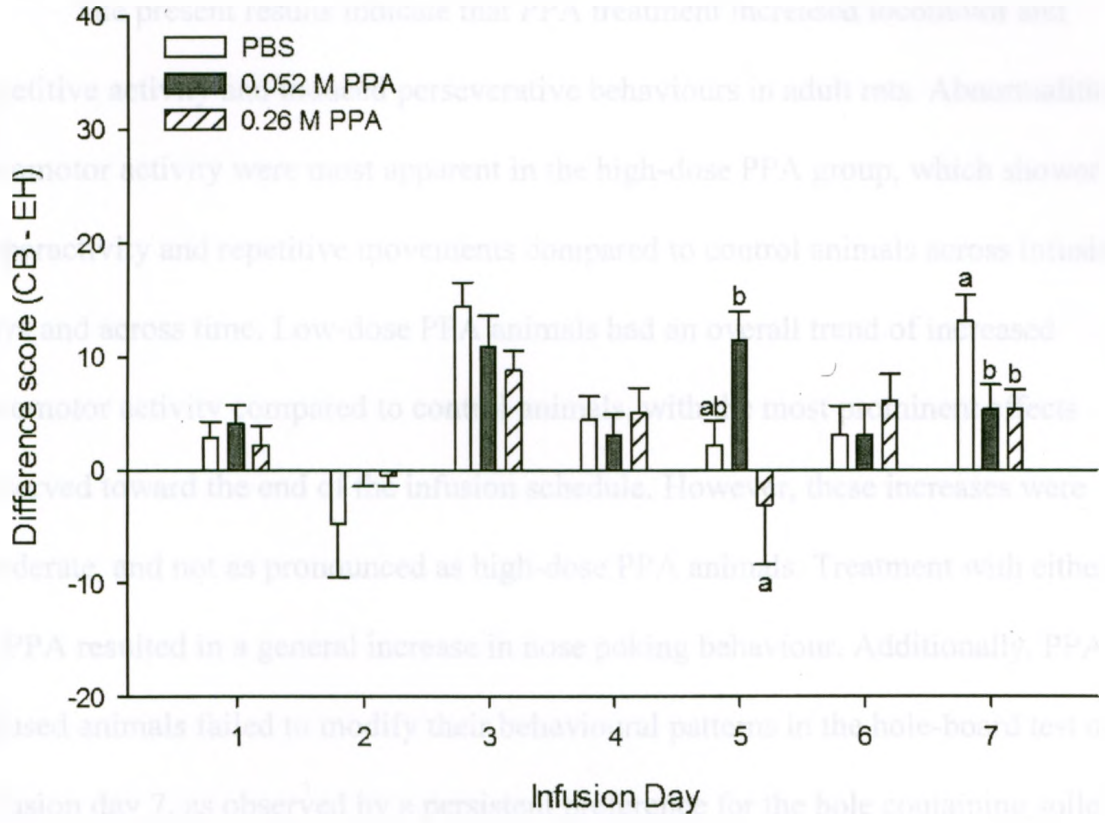

Figure 3.12. Mean difference score (CB-EH) + *SEM* in PBS (n = 11), low-dose PPA (0.052 M, n = 10), and high-dose PPA (0.26M, n = 11) infused animals. Means accompanied by different superscripts (i.e., a or b) indicate a significant difference between treatment groups on that particular infusion day ($p < .05$; no superscripts indicate no significant difference between groups). The total number of nose pokes at the hole with clean bedding (CB) was calculated as a percent of total nose pokes for that particular infusion day for each individual animal. The same was done for the empty hole (EH). The CB percent minus the EH percent is the difference score (CB-EH) that determines the preference for clean bedding (i.e., a positive score indicates a preference for clean bedding) or the preference for an empty hole (i.e., a negative score indicates a preference for an empty hole).

3.4 Discussion

The present results indicate that PPA treatment increased locomotor and repetitive activity and induced perseverative behaviours in adult rats. Abnormalities in locomotor activity were most apparent in the high-dose PPA group, which showed hyperactivity and repetitive movements compared to control animals across infusion days and across time. Low-dose PPA animals had an overall trend of increased locomotor activity compared to control animals, with the most prominent effects observed toward the end of the infusion schedule. However, these increases were moderate, and not as pronounced as high-dose PPA animals. Treatment with either dose of PPA resulted in a general increase in nose poking behaviour. Additionally, PPA-infused animals failed to modify their behavioural patterns in the hole-board test on infusion day 7, as observed by a persistent preference for the hole containing soiled bedding.

Hyperactivity following PPA treatment in the current study, as measured by horizontal activity measures, is consistent with previous work in our laboratory using the same infusion schedule and dose of PPA (0.26M twice daily for seven days) (MacFabe et al., 2007, 2008). However, both PBS and high-dose PPA animals showed considerably greater levels of horizontal locomotion across infusion days than rodents from respective treatment groups in previous experiments. In contrast, vertical and repetitive movement measures in control and high-dose PPA animals were similar to that of earlier work (MacFabe et al., 2008; Thomas et al., 2010). The addition of the hole-board to the automated activity monitors may explain the reason for the overall increase in horizontal movement seen across treatment groups, especially given relevant

olfactory stimuli was present in the wells for most of the infusion schedule. Accordingly, it may be worthwhile to assess locomotor behaviour without the hole-board in low-dose PPA animals in future experiments using this model, as the 0.052 M PPA dose was used for the first time in the current study.

Locomotor behaviour in PPA treated animals decreased over a 30 minute testing session, which is congruent with the known half-life of PPA when administered to rats (Brusque et al., 1999), although the decrease in locomotor behaviour across time could be a result of habituation to the automated activity monitors. Low-dose PPA animals had gradual, albeit moderate, increases in locomotor activity across infusion days, suggesting that chronic low doses of PPA may have led to a decline in physiological mechanisms that metabolize or clear PPA. Such physiologic compensatory mechanisms may include synthesis of metabolizing or pH balancing enzymes, such as propionyl CoA decarboxylase or carbonic anhydrase, respectively (Nguyen et al., 2007; Schlue, Dorner, Rempe, & Riehl, 1991).

In addition to a dose-dependent increase in locomotor activity, PPA treatment also led to a general increase in nose poking behaviour (e.g., a main effect of treatment on nose poking behaviour), that, unlike locomotor activity, was not dose-dependent. However, post-hoc analyses showed that with the exception of infusion day 6 (where low-dose PPA animals made significantly more nose pokes than PBS animals), PPA animals did not nose poke significantly more than PBS animals on any particular infusion day. The interpretation of the general increase in nose poking following PPA treatment is somewhat complex. Nose poking is usually described as a measure of exploratory behaviour, which is influenced by several mitigating factors including level

of arousal, learning and memory, and attention (Berlyne, 1969; Bronson, 1968; File & Wardill, 1975). Others have proposed that nose poking represents an anxiety/escape response or stereotyped behaviour (Makanjuola, Hill, Maben, Dow, & Ashcroft, 1977; Takeda, Tsuji, & Matsumiya, 1998). Another possibility, suggested by Makanjuola and colleagues (1977), is that the overall pattern of nose poking within the hole-board represents exploratory behaviour, whereas repeated nose poking at one particular hole represents repetitive/stereotyped behaviour. Thus, the hole category analysis used in the current study, which assessed the pattern of nose poking within the apparatus, may be a better indicator of overall exploratory activity. All treatment groups showed similar patterns of hole-board exploration, as indicated by the proportion of corner, wall, and centre nose pokes, suggesting that PPA treatment did not alter exploratory behaviour. Although not measured here, the overall increase in nose poking seen in PPA animals could plausibly result from repetitive patterns of nose poking, but hyperactivity cannot be entirely ruled out.

PPA treatment also resulted in a persistent pattern of responding as indicated by hole preference data (i.e, SB-CB and CB-EH difference scores). All treatment groups preferred investigating the hole with soiled bedding over the hole with clean bedding from infusion day 3 through 6. In addition, animals in all treatment groups showed greater interest in the hole with clean bedding over an empty hole on infusion day 3, but this interest declined to infusion day 6 (with the exception of the low dose PPA group on infusion day 5). The most interesting finding is seen on infusion day 7, when the animal's own soiled bedding was used. On infusion day 7, PBS animals reversed their hole preference, showing a preference for the hole with clean bedding over the hole with

soiled bedding, whereas both PPA groups show a preserved interest in the hole with soiled bedding. This is also apparent in the CB-EH difference score for infusion day 7, where PBS animals showed a renewed interest in the hole with clean bedding (comparable to that on infusion day 3 when the bedding was first introduced into the apparatus). In contrast, on infusion day 7 both PPA groups somewhat maintained their levels of interest in the clean bedding hole over the empty hole comparable to infusion day 6.

Although it is clear that PPA treatment resulted in a persistent behavioural response on infusion day 7, it is not entirely clear whether this perseveration reflects a social deficit (and/or an olfactory deficit) or a true cognitive deficit (e.g., in learning and memory). Rodents rely on olfaction to obtain information about their environment, and this is particularly important for social behaviour (Miranda, Ortiz-Godina, & Garcia, 2009). The soiled bedding used in this experiment has inherent olfactory information that is socially relevant. As a result, the persistent pattern of responding in PPA-infused animals could represent a deficit in social information processing. Alternatively, the perseveration may be due to an olfactory deficit in which PPA treated animals cannot distinguish between their own odour and that of a novel conspecific.

The perseverative effect of PPA treatment is also consistent with a cognitive deficit interpretation. The results of the current study are similar to the cognitive impairments in learning and memory observed in previous work using the PPA rodent model (MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011; Shultz et al., 2009). PPA-treated adult or adolescent rats were not impaired during the acquisition phase of the water maze or T-maze, respectively, but were impaired during the reversal phase.

Similarly, PPA-treated animals in the present study were comparable to control animals during the initial exposure to soiled and clean bedding within the hole-board apparatus, but failed to change their behavioural response on infusion day 7 when the environment required a different directional response. The lack of impairment during initial exposure to the hole-board apparatus suggests that the cognitive mechanisms required for processing information within the task were not impaired by PPA.

Importantly, the perseverative behaviours seen in PPA treated animals do not appear to be related to other factors, such as baseline hole preference differences, an inability to distinguish between soiled and clean bedding, or a lack of exploration of the hole-board apparatus. There was no significant difference between treatment groups for hole preference (i.e., SB-CB, SB-EH, and CB-EH difference scores) on infusion days 1 and 2, when all wells were empty, suggesting that there were no baseline differences in hole preference. All treatment groups showed an increase in nose poking on infusion day 3, when clean and soiled bedding was initially placed in the apparatus. Placement of bedding in the centre holes led to a change in the pattern of hole-board exploration for all treatment groups. Specifically, the proportion of centre nose pokes increased dramatically. Additionally, all treatment groups had similar hole preferences from infusion day 3 to 6. Taken together, this suggests that PPA animals were capable of distinguishing between soiled and clean bedding. However, this does not completely rule out that PPA treatment led to more subtle olfactory deficits that made it difficult for animals to differentiate between their own and an unfamiliar male rat's soiled bedding. All treatment groups made a similar proportion of corner, wall, and centre nose pokes across infusion days, suggesting that both PPA groups had hole-board exploration

similar to the PBS animals. Altogether, these results indicate that the perseveration seen in PPA treated animals is not likely confounded by these factors.

PPA has many physiological consequences that alter brain function which can account for the behavioural abnormalities observed in this study. PPA inhibits Na^+/ K^+ ATPase, increases NMDA receptor sensitivity, promotes intracellular calcium release, and elevates nitric oxide, all of which can alter normal neuronal function and transmission (de Mattos-Dutra et al., 2000; Nishiguchi, Hayashi, Shigetomi, Ueda, & Tomita, 1997; Wajner, Latini, Wyse, & Dutra-Filho, 2004; Wyse et al., 1998). Intracellular acidification subsequent to PPA accumulation has widespread effects on neurotransmitter release involving dopamine, glutamate, norepinepherine, and serotonin (Cannizzaro, Monastero, Vacca, & Martire, 2003; Remblier et al., 1999; Severson, Wang, Pieribone, Dohle, & Richerson, 2003), each of which can influence locomotor activity. Repetitive and stereotyped activity has been shown to result from increased levels of dopamine and serotonin in corticostriatal brain circuits, and pH-dependent release of these neurotransmitters may explain the increased nose poking behaviour seen in PPA animals (Langen, Durston, Kas, van Engeland, & Staal, 2011; Langen, Kas, Staal, van Engeland, & Durston, 2011). Increased serotonin may also help explain the perseverative effects of PPA, as serotonin receptor agonists have been shown to impair learning and memory (Ogren et al., 2008) and reduce social behaviour in rodents (Gonzalez, Andrews, & File, 1996).

Another pH-dependent effect of PPA is the rapid and reversible closure of gap junctions, which results in decreased intercellular coupling (Rorig, Klausa, & Sutor, 1996). Gap junctions are necessary for synchronous electrical activity within the brain,

and animal and human studies have shown that gap junctions are important in the regulation of locomotor activity and memory (Juszczak & Swiergiel, 2009). Indeed, studies using connexin 36 knockout mice and gap junction blockers in rodents have shown impairments in spatial memory and cognition and increased stereotypy, repectively (Allen, Fuchs, Jaschonek, Bannerman, & Monyer, 2011; Moore & Grace, 2002). Thus, the increased locomotor behaviour, repetitive nose poking, and perseveration observed in PPA animals could be a result of the closure of gap junctions.

There are several plausible mechanisms for the perseverative effects of PPA treatment. PPA has been shown to result in increased astrogliosis and microglia activation in the hippocampus and white matter suggestive of an innate neuroinflammatory process in the absence of apoptotic neuronal loss (MacFabe et al., 2007). Evidence from neuropathological conditions such as Parkinson's and Alzheimer's disease, which have similar inflammatory responses, suggests that neuroinflammation may play a role in impaired cognitive function (Ferretti & Cuello, 2011; Whitton, 2007). Impaired brain function may also result from increased cytokines, secreted by activated microglia, which is potentially damaging to neurons (Barron, 1995). Consequently, PPA may be inducing a neuroinflammatory response that impairs the function of brain structures important for learning and memory, such as the hippocampus, which may explain the perseveration seen in PPA animals (Leussis & Bolivar, 2006).

In addition, PPA is known to disrupt fatty acid metabolism in the mitochondria via the production of the cytotoxin propionyl Coenzyme A and by inhibiting carnitine function (Brass & Beyerinck, 1988). As a consequence of inhibiting carnitine function,

PPA and other fatty acids can accumulate intracellularly with resultant increases in oxidative stress. Such a diffuse encephalopathic process could lead to cognitive impairments, not unlike those found in the organic acidemias (Wajner et al., 2004). In support of this hypothesis, previous work in our laboratory has shown that PPA treatment results in oxidative stress in widespread brain regions and alters brain lipid and acylcarnitine profiles (MacFabe et al., 2008; Thomas et al., 2010).

PPA and related short chain fatty acids are also capable of inducing extensive alterations in CNS gene expression, presumably through inhibition of histone deacetylase or via modulation of second-messenger transduction systems (Parab et al., 2007). Of particular interest to the current study, short chain fatty acids can induce phosphorylation of cyclic AMP response-element-binding protein (CREB), as demonstrated in both in vitro systems and in rodents using the PPA model (MacFabe et al., 2007; Shah, Nankova, Parab, & La Gamma, 2006). The transcription factor CREB is expressed ubiquitously in brain cells and is known to play a vital role in the epigenetic expression of genes involved in learning and memory (Carlezon, Duman, & Nestler, 2005). Although not measured here, alterations in CREB-dependent memory pathways following PPA treatment may underlie the perseverative behavioural effects seen in the current study.

Results from the present experiment indicate that PPA treatment induced locomotor, repetitive, and perseverative behaviours reminiscent of the symptoms seen in ASD. Those with ASD frequently display hyperactivity and repetitive patterns of behaviour (Matson, Dempsey, & Fodstad, 2009; Murray, 2010), analogous to increased locomotor activity and repetitive nose poking seen in PPA treated rodents. Furthermore,

individuals with ASD often display cognitive dysfunction, such as resistance to change or insistence on sameness and perseverative behaviours (Frith & Happe, 2005; Maes, Eling, Wezenberg, Vissers, & Kan, 2011). The pattern of persistent nose poking despite environmental change within the hole-board apparatus observed in animals treated with PPA is consistent with the cognitive deficits seen in human ASD.

Notably, the proposed underlying mechanisms of PPA-induced behavioural impairments are theoretically linked to ASD. Abnormalities in several neurotransmitters systems have been observed in autistic individuals, including dopamine and serotonin (Chugani, 2004; Previc, 2007). Moreover, serotonin reuptake inhibitors and serotonin and dopamine antagonists are reported to be effective in reducing repetitive thoughts and behaviours in children with ASD (McDougle, Kresch, & Posey, 2000; McPheeters, et al., 2011). An innate neuroinflammatory response, oxidative stress, mitochondrial dysfunction, and disordered fatty acid metabolism have also been found in autism (Chauhan, Chauhan, Brown, & Cohen, 2004; Morgan et al., 2010; Rossignol & Bradstreet, 2008; Vargas et al., 2005) (Wiest et al., 2009).

In summary, ICV infusions of PPA increased locomotor activity and repetitive behaviour, and induced perseveration in adult male rats. The ability of PPA to induce widespread changes in the CNS through intracellular acidification or metabolic dysfunction may offer a potential explanation for the behavioural deficits. Along with previous findings, the current study supports the use of PPA as a model of ASD as behavioural and neuropathological effects following PPA treatment resemble the symptoms and possible underlying pathology of ASD. An increase in short chain fatty acids, via alterations in enteric bacteria or impaired fatty acid metabolism, may be a

plausible environmental factor that triggers or exacerbates ASD in genetically susceptible sub-populations. Further research is needed to clarify which of the diverse physiological effects of PPA and other short chain fatty acids are responsible for ASD-like behaviour in rodents.

3.5 References

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Chapter 4

General Discussion

4.1 General Discussion

The aims of the current studies were to (1) assess repetitive and perseverative behaviours in the hole-board apparatus following PPA treatment and (2) investigate the effects on behaviour of a low-dose of PPA (0.052 M) in comparison to that of a previously used dose of PPA (0.26 M). Both objectives were devised with the intention of further developing the PPA rodent model of ASD. In Chapter 2, locomotor and nose poking behaviour were examined in the hole-board apparatus following PPA treatment. Since this was the first time that the hole-board task was used within the PPA model, all wells within the apparatus remained empty across infusion days. Results showed that central infusions of PPA increased locomotor activity and repetitive nose poking behaviour in a dose-dependent manner. The experiment in Chapter 3 was designed to determine if placement of olfactory stimuli in the centre holes would change the pattern of hole-board exploration, whether animals demonstrated a selective preference for soiled or clean bedding, and most importantly whether animals would adapt their behaviour in response to a change in the environment (i.e., shift their hole preference on infusion day 7). When bedding was located in the centre holes, both PPA groups and the PBS group showed an increase in the proportion of centre nose pokes with a concomitant decrease in the proportion of corner and wall nose pokes suggestive of a change in the pattern of hole-board exploration in response to olfactory stimuli. All treatment groups showed a preference for soiled bedding from an unfamiliar male rat over clean bedding from infusion day 3 through 6. The most interesting finding occurred on infusion day 7, when PPA animals (regardless of dose) showed a preserved interest in the hole containing soiled bedding.

Results from Chapters 2 and 3 showed a dose-dependent increase in locomotor activity following PPA treatment. PPA-infused animals also showed an increase in nose poking behaviour. In contrast, the perseverative effects of PPA occurred regardless of the dose used. Previous work in our lab has also shown that the behavioural response to PPA in rodents is differentially influenced by the timing and spacing of PPA infusions using the same dose (e.g., 1 infusion a week for 5 weeks versus 2 infusions a day for 7 days) (Foley et al., 2008). Investigation of the dose and timing effects of PPA allows for delineation of different behavioural phenotypes that occur as a result of variable PPA exposure. The fact that the rodent model of PPA allows for many behavioural and neuropathological phenotypes is useful given that autism disorders are highly heterogenous and occur across a spectrum of symptom severity (Ratajczak, 2011). Additionally, studying the behavioural response to different PPA regimens might be analogous to the variable exposure to environmental insults seen in human cases of autism. Exposure to infectious agents and environmental toxins during critical developmental periods may trigger ASD in genetically susceptible individuals (Fatemi, 2008). The timing and dose of exposure to these environmental agents may influence the onset, severity, and lifelong modulation of autism (Herbert, 2010).

It is unlikely that an animal model will fully replicate the complexity of a human disease. However, animal models provide a means to explore the neurological, biochemical, and pathological mechanisms underlying disorders such as autism that would be impossible to do in humans. Understanding the common underlying mechanism(s) of a complex neurodevelopmental disorder through an animal model is imperative to the development of better diagnosis, prevention, and treatment. Indeed,

animal models led to effective pharmacological drug therapies used in depression (Willner & Mitchell, 2002). Similar benefits may be derived from the PPA rodent model of autism.

For the PPA rodent model of autism to be useful, effective, and relevant to ASD, it is important to optimize three types of validity within the model: face validity, construct validity, and predictive validity (Crawley, 2007; van der Staay, 2006). Face validity refers to the extent that the symptoms seen in the animal model are similar to those of the human disorder. Some autism symptoms, such as language deficits, may be uniquely human and therefore difficult or impossible to model in rodents. However, other fundamental symptoms of autism, such as social deficits and repetitive behaviour, can be more easily replicated using analogous behaviour in rats. For instance, the current study used total distance traveled as an analog to hyperactivity in autistic human patients. Central infusions of PPA in the current study produced hyperactivity, repetitive nose poking, and perseveration consistent with the symptoms observed in patients with autism (Frith & Happe, 2005; Maes, Eling, Wezenberg, Vissers, & Kan, 2011; Matson, Dempsey, & Fodstad, 2009; Murray, 2010). In addition, previous work has demonstrated social impairment, cognitive deficits, and restricted interests in PPA-treated rats congruent with those seen in ASD (MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011; Shultz et al., 2008, 2009). Taken together, the behavioural effects seen in PPA-treated animals is similar to autism in humans, providing evidence for the face validity of the PPA model of autism. It would be interesting to assess whether PPA-treated animals show impairments in social communication as a means of modelling the language deficits seen in ASD. Crawley (2007) proposed that tests measuring

vocalizations during social interactions or ultrasonic vocalizations by separated pups may be useful behavioural tests to evaluate communication deficits in rodents. Future studies of the PPA rodent model using communication –related tests would address another core symptom of ASD, further validating the model.

In addition to face validity, an optimal animal model should have construct validity and predictive validity (van der Staay, 2006). Construct validity refers to the extent that the animal model has a sound theoretical rationale based on the similarity of the animal model to the underlying causes of the disease. Predictive validity refers to the extent that the animal model shows an expected response to treatments used in the human disorder. For instance, if a clinical drug is effective in the treatment of autism, then the same drug should show a similar response within the animal model.

The evaluation of the construct validity of the PPA rodent model of autism is limited because of a lack of an existing comprehensive theory of the aetiology of autism. However, the similarities in the neuropathological, biochemical, and proposed mechanistic effects of PPA treatment and human ASD could represent a similar underlying aetiological process. For instance, PPA promotes intracellular calcium release, elevates nitric oxide, and increases the synthesis and release of dopamine and serotonin, all of which may help explain the increase in locomotor activity and repetitive behaviour observed in the current study (Nishiguchi, Hayashi, Shigetomi, Ueda, & Tomita, 1997; Wajner, Latini, Wyse, & Dutra-Filho, 2004). Similarly, individuals with ASD have been shown to have calcium signalling abnormalities, elevated plasma levels of nitric oxide, and aberrations in dopamine and serotonin neurotransmitter systems (Chugani, 2004; Gargus, 2009; Previc, 2007; Sweeten, Posey, Shankar, & McDougle,

2004). Examination of brain tissue in rodents following PPA administration also bears resemblance to findings in human ASD cases. PPA treatment results in neuroinflammation, impaired glutathione metabolism, and altered fatty acid profiles reminiscent of the activated microglia, increased oxidative stress, and disordered mitochondrial fatty acid metabolism observed in human ASD (James et al., 2009; MacFabe et al., 2007; Rossignol & Bradstreet, 2008; Sajdel-Sulkowska, Lipinski, Windom, Audhya, & McGinnis, 2008; Thomas et al., 2010; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). Furthermore, PPA has both electrophysiological and gastrointestinal effects which can account for the high co-morbidity of seizures and intestinal disorders in ASD (MacFabe et al., 2007; Mitsui, Ono, Karaki, & Kuwahara, 2005; Tuchman, Moshe, & Rapin, 2009; White, 2003).

One of the most common and consistent neuropathological findings in the brain of those with ASD is reduced number and size of Purkinje cells in the cerebellum (Bauman & Kemper, 2005; Fatemi, et al., 2002; Palmen, van Engeland, Hof, & Schmitz, 2004). Cerebellar dysfunction has been linked to reduced exploration and stereotyped behaviour in MRI studies of children with autism (Pierce & Courchesne, 2001), and repetitive behaviour and increased activity has been observed in a mouse model of Purkinje cell loss (Martin, Goldowitz, & Mittleman, 2010). Furthermore, prenatal exposure to valproic acid in rodents, a derivative of PPA, reproduces the cerebellar abnormalities associated with ASD (Ingram, Peckham, Tisdale, & Rodier, 2000). Given the ability of PPA to induce repetitive and stereotyped behaviour in the current study, it would be interesting to investigate whether Purkinje cell atrophy occurs in the

cerebellum of PPA treated rodents. This would support further the construct validity of the PPA model.

Preliminary evidence exists for the predictive validity of the PPA model. Dopamine antagonists have been shown to be effective in reducing some of the behavioural symptoms in autism (McPheeters et al., 2011). Similarly, work in our lab has shown a decrease in PPA-induced locomotor behaviour as a result of treatment with dopamine receptor antagonists (Martins et al., 2008), providing initial support for the predictive validity of the model. Assessment of the effects of carnitine supplementation would be a reasonable next step in addressing the predictive validity of the PPA rodent model. Carnitine is essential for the proper metabolism of fatty acids within the mitochondria (Jones, McDonald, & Borum, 2010), and a relative carnitine deficiency has been observed in blood plasma of autistic patients (Filipek, Juranek, Nguyen, Cummings, & Gargus, 2004). A randomized double-blind clinical trial of L-carnitine to treat ASD resulted in significant improvements in several clinical measurements of autism severity (Geier et al., 2011). Since studies in our lab have shown that PPA treatment in rodents results in altered plasma acylcarnitine profiles similar to those in ASD (Thomas et al., 2010), future experiments using carnitine treatment in PPA-treated rats would be useful in assessing the predictive validity of the PPA model.

PPA and related short chain fatty acids may be a possible common underlying mechanism behind the multi-factorial aspects of ASD. Because PPA has the ability to cross both the gut-blood and blood-brain barriers, it is a plausible environmental link between brain, gastrointestinal, and dietary factors seen in ASD (Karuri, Dobrowsky, & Tannock, 1993). Rather than PPA being a direct cause of autism per se, elevated levels

of PPA early in development may be an environmental risk factor which can trigger or exacerbate ASD or ASD related behaviours in genetically susceptible populations. It is important to note that there are no studies directly measuring PPA and related metabolites in ASD patients. However, there is some indirect evidence suggestive of increased PPA in autism, such as impaired fatty acid metabolism (Clark-Taylor & Clark-Taylor, 2004) , relative carnitine deficiency (Filipek et al., 2004), and reductions in essential polyunsaturated fatty acids levels (Vancassel et al., 2001). Moreover, increases in clostridial bacteria species, which produce PPA, have been observed in the gut of autistic patients (Finegold et al., 2002; Parracho, Bingham, Gibson, & McCartney, 2005).

In conclusion, the present studies provide further support for the PPA rodent model of autism. Animals infused with PPA showed hyperactivity, increased repetitive behaviour, and perseveration consistent with the symptoms seen in human ASD. It is unclear which of the diverse effects of short-chain fatty acids are responsible for the ASD-like behaviour in rodents, and future studies are needed to address this question. Nevertheless, administration of PPA in rodents does show promise as an animal model to link the disparate behavioural, metabolic, and gastrointestinal findings in ASD.

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